EVALUATION OF WOUND HEALING EFFECT OF AQUEOUS STEM EXTRACT OF *Zanthoxylum rhetsa* (Roxb.) DC. ON WISTAR RATS

A Dissertation Submitted to

THE TAMIL NADU Dr. M. G. R. MEDICAL UNIVERSITY

CHENNAI-600 032

In partial fulfillment of the requirement for the award of the Degree of

MASTER OF PHARMACY

IN

PHARMACOLOGY

OCTOBER-2017

DEPARTMENT OF PHARMACOLOGY

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KOVAI ESTATE, KALAPPATTI ROAD,

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S. Parthiban

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ABBREVIATIONS

ABBREVIATIONS	FULL FORM
AQZR	Aqueous stem Extract of *Zanthoxylum rhetsa*
ABTS	2,2'-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid)
DMSO	Dimethyl Sulfoxide
DPPH	1, 1-diphenyl-2-picrylhydrazyl
GAE	Gallic acid Equivalent
QE	Quercetin Equivalent
WHO	World Health Organization
NO	Nitric Oxide
ABS	Absorption
MTCC	Microbial Type Culture Collection
µg	Micro gram
Fig	Figure
ºC	Degree centigrade
mm	Milli meter
NCIM	National Collection of Industrial Micro organisms

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ABSTRACT

The present investigation has been undertaken to study the wound healing properties of aqueous extract of *Zanthoxylum rhetsa*. The plant *Zanthoxylum rhetsa* has a long history in herbal medicine in many countries. Experiments were conducted following standard procedures. The extracts were evaluated for their *in vitro* antioxidant, antimicrobial and total phenol and flavonoid content. The AQZR ointment were administered topically, for evaluating the wound healing potential in excision wound model for twenty one days. Povidone iodine ointment was used as a standard for wound healing in excision wound model. Extract treated group showed *in vitro* antioxidant, antimicrobial properties compared with standard and control. AQZR exhibited similar *in vivo* wound healing activity that of the standard but with lesser magnitude. The result may be attributed to the phytoconstituents such as flavonoids and phenolics present in it which may be due to their individual or cumulative effect that enhanced wound healing and provided scientific evidence to the ethnomedicinal futures of *Zanthoxylum rhetsa*. These findings could justify the inclusion of this plant in the management of wound healing.

Keywords: AQZR, Wound healing potential, Phytoconstituents.

1. INTRODUCTION

1.1 HERBAL MEDICINES [1][2][3][4]

Herbal medicines which formed the basis of health care throughout the world since the earliest days of mankind are still widely used, and have considerable importance in international trade. Recognition of their clinical, pharmaceutical and economic value is still growing, although this varies broadly between countries.

Medicinal plants are important for pharmacological research and drug development, not only when plant constituents are used directly as remedial agents, but also as starting materials for the synthesis of drugs or as models for pharmacologically active compounds. Regulation of development and exportation is therefore essential, together with international cooperation and coordination for their conservation so as to ensure their availability for the future.

The United Nations Convention on Biological Diversity states that the conservation and sustainable use of biological diversity is of critical importance for meeting the food, health and other needs of the growing world population, for which purpose access to and sharing of both genetic resources and technologies are essential.

Legislative controls in respect of medicinal plants have not evolved around a structured control model. There are different ways in which countries define medicinal plants or herbs or products derived from them, and countries have adopted various approaches to licensing, dispensing, manufacturing and trading to ensure their safety, quality and efficacy.

Despite the use of herbal medicines over many centuries, only a relatively small number of plant species has been studied for possible medical applications. Safety and efficacy data are available for an even smaller number of plants, their extracts and active ingredients and preparations containing them.

India is a birth place of indigenous medicine such as Siddha, Ayurveda and Unani where many herbs have been used for treatment of human ailments. About 65% of

total global population remains dependent on traditional medicines for their primary healthcare. Herbs are occupying a comeback and an Herbal Renaissance is blooming across the world. They have been evidently prized for their medicinal, flavoring and aromatic qualities for centuries, yet for a while they were over shadow by synthetic products of modern civilization. Folk medicine is generally defined as traditional medicine that is practiced by non-professional healers or embodied in local custom or lore, generally involving the uses of natural and especially herbal remedies. The World Health Organization (WHO) defines traditional medicine as "the health practices, approaches, knowledge and believes incorporating plant, animal and mineral-based medicines, spiritual therapies, manual techniques and exercise, applied singularly or in combination to treat, diagnose and prevent illness or maintain well-being." Treatments are specific to a particular ailment and may include the power of rare, dancing, sweet baths, massage, medicinal herbs, hot and cold foods or other means not practiced in modern medicine.

Once having realized their sources and adverse effects, people are going to nature with hopes of safety and security. The rich treasure of herbal drugs is forming a boon for our society. Plant derived compounds, apart from their nutritive values, could serve as important therapeutic weapons to fight various human and animal diseases, thereby making them indispensable in traditional medicine for treating a number of diseases. Plant drugs, popularly known as herbal medicines have since been unabatedly used to that various diseases. The major challenge is to protect traditional knowledge and will prove to be a beneficial asset to our human surrounding. For all the ailments herbal formulations are proved to be effective without any side effects commonly seen with allopathic drugs.

1.2 INFLAMMATION [5][6]

Inflammation (Latin, inflammatio) is part of the complex biological response of body tissues to harmful stimuli, such as pathogens, damaged cells, or irritants. Inflammation is a protective response that involves immune cells, blood vessels, and molecular mediators. The purpose of inflammation is to eliminate the initial cause of cell injury, clear out necrotic cells and tissues damaged from the original insult and the inflammatory process, and to initiate tissue repair.

The classical signs of acute inflammation are pain, heat, redness, swelling, and loss of function. Inflammation is a generic response, and therefore it is considered as a mechanism of innate immunity, as compared to adaptive immunity, which is specific for each pathogen.

Inflammation is tightly regulated by the body. Too little inflammation could lead to progressive tissue destruction by the harmful stimulus (e.g. bacteria) and compromise the survival of the organism. In contrast, chronic inflammation may lead to a host of diseases, such hay fever, periodontitis, atherosclerosis, rheumatoid arthritis, and even cancer (e.g., gallbladder carcinoma). Inflammation is therefore normally closely regulated by the body.

Inflammation can be classified as either acute or chronic.

Acute inflammation is the initial response of the body to harmful stimuli and is achieved by the increased movement of plasma and leukocytes (especially granulocytes) from the blood into the injured tissues. Prolonged inflammation, known as chronic inflammation, leads to a progressive shift in the type of cells present at the site of inflammation and is characterized by simultaneous destruction and healing of the tissue from the inflammatory process.

Inflammation is not a synonym for infection. Infection describes the interaction between the action of microbial invasion and the reaction of the body's inflammatory defensive response.

Table:1 Comparison Between Acute and Chronic Inflammation [7]

Factors	Acute Inflammation	Chronic Inflammation
Causative agent	Bacterial pathogens, injured tissues	Persistent acute inflammation due to non-degradable pathogens, viral infection, persistent foreign bodies, or autoimmune reactions.
Major cells involved	Neutrophils (primarily), basophils (inflammatory response), and eosinophil's (response to helminth worms and parasites),	Mononuclear cells (monocytes, macrophages, lymphocytes, plasma cells), fibroblasts
Primary mediators	Vasoactive amines, Eicosanoids.	IFN-γ and other cytokines, growth factors, reactive oxygen species, hydrolytic enzymes
Onset	Immediate	Delayed
Duration	Few days	Up to many months, or years
Outcomes	Resolution, chronic inflammation	Tissue destruction, fibrosis, necrosis

Acute inflammation is a short-term process, usually appearing within a few minutes or hours and begins to cease upon the removal of the injurious stimulus. It is characterized by five cardinal signs: An acronym that may be used to remember the key symptoms is "PRISH" for Pain, Redness, Immobility (loss of function), Swelling and Heat.

The traditional names for signs of inflammation come from Latin:[8][9][10]

- Dolor (pain)
- Calor (heat)
- Rubor (redness)
- Tumor (swelling)
- Functiolaesa (loss of function)

The first four (classical signs) were described by Celsus (ca. 30 BC–38 AD), while loss of function was added later by Galeneven though the attribution is disputed and the origination of the fifth sign has also been ascribed to Thomas Sydenham and Virchow.

Redness and heat are due to increased blood flow at body core temperature to the inflamed site; swelling is caused by accumulation of fluid; pain is due to the release of chemicals such as bradykinin and histamine that stimulate nerve endings. Loss of function has multiple causes.

Acute inflammation of the lung (pneumonia) does not cause pain unless the inflammation involves the parietal pleura, which does have pain.

Inflammatory disorders [11]

Inflammatory abnormalities are a large group of disorders that underlie a vast variety of human diseases.

Examples of disorders associated with inflammation include:

- ❖ Acne vulgaris
- ❖ Asthma
- ❖ Autoimmune diseases

- Auto inflammatory diseases
- Celiac disease
- Chronic prostatitis
- Glomerulonephritis
- Hypersensitivities
- Inflammatory bowel diseases
- Reperfusion injury
- Rheumatoid arthritis
- Sarcoidosis
- Transplant rejection
- Vasculitis
- Interstitial cystitis

1.3 WOUND HEALING ACTIVITY [12]

Wounds are inescapable events in life. Wounds may arise due to physical, chemical or microbial agents. Wound healing involves a complex series of interactions between different cell types, cytokine mediators, and the extracellular matrix. The phases of normal wound healing include hemostasis, inflammation, proliferation and remodeling. Each phase of wound healing is distinct, although the wound healing process is continuous, with each phase overlapping the next. Because successful wound healing requires adequate blood and nutrients to be supplied to the site of damaged tissue.

CLASSIFICATION OF WOUNDS

Wounds are classified as open wounds and closed wounds on the basis of underlying cause of wound creation and as acute and chronic wounds on the basis of physiology of wound healing

(a) Open Wound: [13]

Though the open wound blood escapes the body and bleeding is clearly visible.

Open wound is further classified as:

- Incised Wounds:

  It is an injury with no tissue loss and minimal tissue damage. It is caused by a sharp object such as knife. Bleeding in such cases can be profuse, so immediate action should be taken.

- Abrasions or superficial Wounds:

  It is caused by sliding fall on to a rough surface. During abrasion the topmost layer of the skin i.e. epidermis is scraped off that exposes nerve ending resulting in a painful injury. Blood loss similar to a burn can result from serious abrasions.

- Laceration wound or tears Wounds:

  This is a nonsurgical injury in conjunction with some type of trauma, resulting in tissue injury and damage.

- Puncture Wounds:

  They are caused by some object puncturing the skin, such as needle or nail. Chances of injection in them are common because dirt can enter into the depth of wound.

- Gunshot Wounds:

  They are caused by a bullet or similar driving into or through the body.

- Penetration Wounds:

  Penetration wounds are caused by an object such as knife entering and coming out from the skin.

(b) Closed Wounds:

In closed wounds blood escapes the circulating system but remain in the body. It includes contusion or bruises, heamatomas or blood tumor, crush injury etc.

- Contusions or bruises:

  Bruises are caused by a blunt force trauma that damage tissue under the skin.

- Hematomas or blood tumor:

  They are caused by damage to a blood vessel that consequently causes blood to collect under the skin.

- Crush injury:

  Crush injury is caused when great or extreme amount of force is applied on the skin over long period of time.

- Acute Wounds:

  An acute wound is a tissue injury that normally proceeds through an orderly and timely reparative process that results in sustained restoration of anatomic and functional integrity. Acute wounds are usually caused by cuts or surgical incisions and complete the wound healing process within the expected time frame.

- Chronic wounds:

  Chronic wounds are wounds that have failed to progress through the normal stages of healing and therefore enter a state of pathologic inflammation chronic wounds either require a prolonged time to or recur frequently. Local infection, hypoxia, trauma, foreign bodies and systemic problems such as diabetes mellitus, malnutrition, immunodeficiency or medications are the most frequent causes of chronic wound.

FACTORS AFFECTING WOUND HEALING [14]

- Improper diet.
- Infection at the wound site.
- Insufficient oxygen supply and tissue perfusion to the wound area.
- Drugs.
- Elderly age.
- Diabetes and other diseases conditions.

Wound healing is normal biological process in the human body. Many factors can adversely affect this process and lead to improper and impaired wound healing.

Improper Diet:

Wound healing is an anabolic process that requires both energy and nutritive substrates. It is reported that serum albumin level of 3.5mg/dl or more is necessary for proper wound healing. Protein is essential for collagen synthesis on wound site. A state malnutrition may provide an inadequate amount of protein and this can decreased the rate of collagen synthesis, wound tensile strength or increased chance of infection.

Infection at the wound site:

Wound infection is probably the most common reason of impaired wound healing by *Streptococcus aereus, Streptococcus pyrogens, Escherichia coli,* and *Pseudomonas aeruginosa.*

Insufficient Oxygen Supply and Tissue Perfusion to the Wound area:

Adequate blood supply and tissue perfusion is extremely important for wound healing. Excessive pain, cold and anxiety can cause local vasoconstriction and increased healing time. Smoking and use of tobacco decreased tissue perfusion and oxygen tension in wound.

Drugs:

Many drugs are known to impair wound healing. Chemotherapeutic drugs are used in cancer are the largest group well known to delay wound repair. Systemic glucocorticoids interfere normal healing process by reducing collagen synthesis and fibroblast proliferation.

Elderly Age:

Elderly age is found to be associated with delay wound healing. It is reported that the fibroblast growth and activity diminishes and collagen production, wound contraction is slow in older individuals

Diabetes and Other Diseases:

Diabetic patients are more susceptible to wound healing. In the studies wound infection rate was found 11% higher in diabetic patients than in general patient's populations. Acute and chronic liver diseases also associated with delay wound healing. Patients with altered immune functions have an increased susceptibility to wound infection.

PHASES INVOLVED IN WOUND HEALING:

- The Inflammatory phase
- Fibroblastic phase
- Epithelialization phase
- Proliferative phase
- Contraction phase
- Remodeling phase

THE INFLAMMATORY PHASE: [15]

The inflammatory phase starts immediately after the injury that usually last between 24 and 48hrs and may persist for up to 2 weeks in some cases the inflammatory phase launches the haemostatic mechanisms to immediately stop blood loss from the wound site. Clinically recognizable cardinal sign of inflammation, rubor, calor, tumor, dolor and functionless appear as the consequence. This phase is characterized by vasoconstriction and platelet aggregation to induce blood clotting and subsequently vasodilatation and phagocytosis to produce inflammation at the wound site.

FIBROBLASTIC PHASE: [16]

The second phase of wound healing is the fibroblastic phase that lasts upto 2 days to 3 weeks after the inflammatory phase. This phase comprises of three steps viz. granulation, contraction and epithelialization in the granulation step fibroblasts form a bed of collagen and new capillaries are produced. Fibroblast produces a variety of substances essential for wound repair including glycosaminoglycans and collagen. Under the step of contraction wound edges pull together to reduces the defects in the third step epithelial tissues are formed over the wound site.

EPETHELIALIZATION PHASE: [17]

Epithelial cell migration is one of the vital processes of wound healing. The stem cells of epithelium must detach from the edges of the wound and migrate into wound. Normally dermal basal cells adhere to each other and to the underline basal layer of the dermis. Following mobilization, epithelial cells begin to enlarge and migrate down and across the wound. Transected hair follicles also contribute to the number of migrating epithelial

cells. Epithelial cell migrating across wound usually along the basal lamina or fibrin deposits, this phenomenon is called contact guidance and is an important factor in epithelial migration. Epithelial migration is followed by increased mitosis of epithelium. Recent evidence suggests that a water soluble heat labile substance called chalcone which is secreted at the wound site is responsible for regulation for mitosis.

PROLIFERATIVE PHASE: [18]

Proliferative phase (2 days to 3 weeks) includes:

- Granulation stage: Fibroblasts lay bed of collagen fills defect and produces new capillaries.
- Contraction stage: Wound edges pull together to reduce defect.
- Epithelialization stage: Crosses moist surface cell travel about 3cm from point of origin in all directions.

CONTRACTION PHASE: [19]

Wound contraction is caused by the action of differentiated fibroblasts (myofibroblasts) in the granulation tissue, which contain filaments of smooth muscle action. Contraction of these fibroblasts makes the wound margins move toward the center of the wound. Wound contraction started sooner in ponies than in horses and it was significantly more pronounced in ponies. Additionally, it was significantly more pronounced in body wounds compared with the limb wounds. As a result, intention wound healing was significantly faster in ponies than in horses, and significantly faster in body wounds than in metatarsal wounds. Histology showed that myofibroblasts were more organized in the wounds of the ponies: the myofibroblasts in the newly formed granulation tissue were transformed into a regularly organized pattern within 2 weeks, in which the cell were orientated perpendicular to the vessels and parallel to the wound surface. This appears to be a more favorable condition for wound contraction to occur. In the horses, myofibroblast organization took much longer. No differences were found in the number of fibroblasts, the amount of smooth muscle action and collagen. Further research was performed to investigate whether the differences in wound contraction between horses and ponies were caused by differences in the inherent contraction capacity of fibroblasts or the local environment of the fibroblasts. It was found that no differences existed in the inherent contraction capacity of fibroblasts from ponies and horses *in vitro*. However the

level of transforming growth factor, the most important instigator of wound contraction, was significantly higher in the granulation tissue of pony wounds compared with horse wounds.

REMODELING PHASE: [20]

This phase last for 3 weeks to 2 years. New collagen is formed in this phase. Tissue tensile strength is increased due to intermolecular cross-linking of collagen via vitamin-C dependent hydroxylation. The scar flattens and scar tissues become 80% as a strong as the original.

The wound healing activities of plants have since been explored in folklore. Many ayurvedic herbal plants have a very important role in the process of wound healing. Plants are more potent healers because they promote the repair mechanisms in the natural way. Extensive research has been carried out in the area of wound healing management through medicinal plants. Herbal medicines in the wound management involve disinfection, debridement and providing a moist environment to encourage the establishment of the suitable environment for natural healing process.

ACTIVITIES LEADING TO WOUND HEALING

(a) **Anti-inflammatory Activity:**

The acute inflammatory response during the early stages of injury generates factors that are essential for tissue growth and repair. When prolonged, however, chronic inflammation can to be detrimental, preventing wound remodeling and matrix synthesis, leading to delay in wound closure and an increase in wound pain. Thus, it is possible that an anti-inflammatory effect could facilitate wound healing and improve patient comfort, although traditional texts and animal studies indicate that extracts exert an anti-inflammatory effect.

(b) **Antioxidant Effect:**

The production of free radicals at or around the wound may contribute to delay in wound healing through the destruction of lipids, proteins, collagen, proteoglycan and hyaluronic acid. Agents that demonstrate a significant antioxidant activity may, therefore, preserve viable tissue and facilitate wound healing.

(c) **Antimicrobial Activity:**

Wound healing can also be delayed when microoganisms are present in large enough numbers. Therefore, reducing the bacterial load of a wound may be necessary to facilitate wound healing as well as to reduce local inflammation and tissue destruction. An ideal agent for the prevention and control of wound infection would therefore be one that directly destroys the pathogens while also stimulate immune activity.

(d) **Analgesic Activity:**

The open wounds can generate pain and subsequent disability, it is important that the dressing applied does not increase pain, and if possible, it should lessen the pain.

2. PLAN OF WORK

1. Review of Literature.
2. Selection Collection and Authentication of Plant Material.
3. Extraction of plant material stem with Water.
4. Preliminary Phytochemical analysis
5. Quantification of total Phenol and Flavonoid content.
6. *Invitro* antioxidant study
    - DPPH radical scavenging assay.
    - ABTS radical cation assay.
    - Nitric oxide scavenging assay.
7. *In vitro* antimicrobial study.
    - Antibacterial.
    - Antifungal.
8. Pharmacological study.
9. Histopathological study.
10. Statistical analysis.

3. LITERATURE REVIEW

1. **M.T.Rahman, *et al.,*** Antinociceptive and Antidiarrheal activity. The methanolic extract of the *Zanthoxylum rhesta* (Roxb.) DC**.** Stem bark. Significantly reduced the abdominal contraction induced by acetic acid and the diarrheal episodes induced by castor oil in mice. [21]
2. **Md.Khrul Islam, *et al.,*** Anti-nociceptive and Antioxidant activity. Different parts of the medicinal plant *Zanthoxyllum budrunga* wall seeds (ZBSE) to evaluate its antinociceptive and antioxidant potential. [22]
3. **T.G.Nagaraja *et al.,*** *In-vitro* Antibacterial properties. *Invitro* screening of antibacterial properties of *Z.rhesta* (Roxb.) DC. of bark & stem showed antibacterial properties. [23]
4. **T.G.Nagaraja *et al.,*** *In-vitro* evaluation of Anti-fungal properties. *Invitro* screening of anti-fungal properties of root, stem & bark of *Zanthoxylum rhesta* (Roxb.) DC**.** possess good fungicidal properties against *alternaria alternate, aspergillus niger, fusarrium oxysporum, cladosporum sp., macrophoma phaseolina and thrichoderma viridi*. [24]
5. **Aparna Saraf *et al.,*** Chemical profile studied. To establish the chemical fingerprint of various secondary metabolites of *Zanthoxylum rhesta* (Roxb.) DC**.** A medicinally important plant. HPTLC profiles of various individual secondary metabolites were done and profiles developed for authentication. This study was useful in differentiating the species from the adulterant and also act as biomarker for this plant in the pharmaceutical industry. [25]
6. **Anwarul Islam abu sayeed *et al.,*** Brain shrimp lethality bioassay. The cytotoxic activity of free terpenes isolated from the bark of *Zanthoxylum budrunga* wall used in folk medicine in Bangladesh, was evaluated by brine shrimp lethality bioassay. [26]
7. **Suresh lalitharani *et al.,*** G.C-MS analysis. The investigation deals with GC-MS analysis of ethanolic extract of the spine of the *Zanthoxylum rhesta* (Roxb.) DC. plant fifteen compound were identified. [27]
8. **S.Lalitharani,*et al.,*** Pharmacognostic studies. Investigation deals with pharmacognostic studies, phytochemical studies and fluorescence analysis. The present investigation deals with the pharmacognostic studies of the spine of the said

plant. Pharmacognostic studies include microscopic, physicochemical constants, fluorescence analysis and preliminary phytochemical evaluations. [28]

9. **Temin payum *et al.,*** Antioxidant potencial .The methanolic extract of shoot were evaluated for total phenolic content (folin-ciocalteu's method), total flavonoids content (colorimetric method) and antioxidant potential (DPPH and ABTS). [29]
10. **R.Rajashri Naik, *et al.,*** GC-MS Analysis and biological evaluation of Essential oil of *Zanthoxylum rhetsa*(Roxb.) DC pericarp. The present study reports the chemical composition, antioxidant, antimicrobial, antidiarrheal as well as the spasmolytic activity of *Zanthoxylum rhetsa*(Roxb)DC essential oil, fractionated oil and its principal constituent (Terpinen-4-ol) The constituents of essential oil were characterized by GC-FID and GC-MS. The pharmacological and biological activities of oil, its fraction and principal constituent were carried out *in-vivo* and *in-vitro.* The study suggests that the oil and its main active constituent (Terpinen-4-ol) of the studied plant would have high potential in the treatment of stress and gastro intestinal diseases. [30]
11. **Rajashrinaik, *et al.,*** GC-FID Analysis of fatty acids and biological Activity of *Zanthoxylum rhetsa* (Roxb.) DC seed oil. The fatty acid content and composition of fixed oil from *Zanthoxylum rhetsa* seeds was determined. The seeds were found to contain about -19.5% of crude foxed oil on dry weight basis. Fatty acids were converted in to methyl esters and analyzed GC-FID. Ten fatty acids were identified using GC-FID. The major monosaturated and saturated fatty acids were oleic acids and palmitic acids respectively, whereas the alpha linolenic acid and linolenic acid were poly unsaturated fatty acid. Fixed oil exhibited significant free radical scavenging activity which was measured using DPPH, and is also known to inhibit the gastrointestinal motility significantly. [31]
12. **A Ramesha Rao, *et al.,*** Chemoprotective influence of *Zanthoxylum* sps. On hepatic carcinogen metabolizing and antioxidant enzymes and skin papillomagenesis in murine model. In the present study, the putative of pericarp of dried fruit *Zanthoxylum* (Rutaceae Family), a common spice additive in Indians west coast cuisines, in protecting against carcinogenesis has been reported. Result showed a significant reduction in tumor incidence from 68% to 36% ($p<0.05$); as well as, a reduction in tumor burden from effective mouse from 3.87 to 0.72 ($p<0.01$).

Cumulative, the findings strongly suggest cancer chemo preventive potential of *Zanthoxylum* sps. [32]

13. **S.K.Nazrul islam *et al.,*** Constituents and cytotoxicity of *Zanthoxylum rhetsa* stem bark. Xanthyletin and sesamin have been isolated from petroleum ether extract of the stem bark of *Zanthoxylum rhetsa*. The petroleum ether extract showed cytotoxicity on brine shrimp nauplii. [33]
14. **Vijitr Udeye *et al.,*** Characteriaztion of the Essential oil and Fatty oil from Makhwaen fruit (*Zanthoxylum rhetsa* (Roxb.) DC). [34]
15. **Khozirah shaari *et al.,*** Bioactive constituents of *Zanthoxylum rhetsa*. Bark and its Cytotoxicity Potential against B16-F10 Melanoma cancer and Normal Human Dermal Fibroblast (HDF) Cell lines. This plant has been predominantly used by Indian tribes for the treatment of many infirmities like diabetes, inflammation, rheumatism, toothache and diarrhea. The presence of bioactive lignans and alkaloids were suggested to be responsible for the cytotoxicity property of Z.*rhetsa* bark against B16-F10 cells. [35]
16. **V.S. Rana *et al.,*** Volatile constituents of the seed coat of *Zanthoxylum rhetsa*(Roxb.) DC. The chemical composition of the volatile oil of *Zanthoxylum rhetsa* seed coat was analyzed by GC and GC/MS. Thirty four compounds, accounting for 87.4% of the oil were identified. The major compounds were terpinen-4-ol(32.1%), alpha-terpineol(8.2%), sabinene (8.1%), and 2-undecanone(7.1%). [36]

4. PLANT PROFILE [37][38][39]

Figure:1 *Zanthoxylum rhetsa* (Roxb.) DC.

INTRODUCTION:

General Information of *Zanthoxylum rhetsa*(Roxb.) DC.

A genus of herbs belongs to the family **Rutaceae** well distributed in India, Bangladesh, Bhutan, China etc.

Fifteen species are found naturalized in India and Bangladesh.

1. ***Zanthoxylum rhetsa*(Roxb.)DC.**
2. ***Zanthoxylum nitidum***
3. ***Zanthoxylum chalybeum***
4. ***Zanthoxylum americanum.***
5. ***Zanthoxylum oxyphyllum***
6. ***Zanthoxylum armatum***
7. ***Zanthoxylum piperitum***
8. ***Zanthoxylum simulans***
9. ***Zanthoxylum bungeanum max***
10. ***Zanthoxylum sehinifolium***
11. ***Zanthoxylum nitidum*Roxb**
12. ***Zanthoxylum armatum* DC(or) alatumRoxb**
13. ***Zanthoxylum limonella***
14. ***Zanthoxylum avicennae* (Lamk) DC**
15. ***Zanthoxylum acanthopodium* DC**

LATIN BINOMIAL & VERNACULAR NAMES:

Source	:	***Zanthoxylum rhetsa* (Roxb.) DC.**
Synonym	:	Budrunga
Family	:	Rutaceae

VERNACULAR NAMES:

General Name	-	Kasabang(llk),kayatena(tag)
English Name	-	Indian prickly ash-tree.
Botanical Name	-	Zanthoxylumrhetsa (Roxb.) DC.
Tamil Name	-	Veerasangi
Sanskrit	-	Lakhuvalka(la), Asvaghra.
Malayalam	-	Mulilam, Mulliyllam, Karimurikka.
Kannada	-	Aramadala,Arampala,Jammina
Hindi Name	-	Badrang.

SCIENTIFIC CLASSIFICATION:

Kingdom	-	Plantae
Phylum	-	Tracheophyta
Division	-	Eukaryota
Class	-	Spematopsida
Order	-	Sapindales
Suborder	-	Rutineae
(Unranked)	-	Angiosperms, Eudicots, Rosids
Family	-	Rutaceae
Subfamily	-	Rutoideae
Tribe	-	Zanthoxyleae
Genus	-	Zanthoxylum
Species	-	Zanthoxylum rhetsa

5. METHODOLOGY

5.1. PLANT COLLECTION AND AUTHENTICATION

The stem part of the plant *Zanthoxylum rhetsa* (Roxb) DC was collected from Palakkad District of Kerala and authenticated from the Plant Anatomy Research Center, Tambaram, Chennai, Tamil Nadu. The authentication certificate number is PARC/2016/3324. Soon after collection, the stems were cleaned and shade dried. After drying, these stems were crushed to a coarse powder, stored in air tight plastic container for further use.

5.2. EXTRACTION OF THE PLANT MATERIAL [40]

The coarsely powdered stem (200gm) were taken in a round bottom flask and with water for 48hours at room temperature. After extraction the extracts were evaporated or concentrated by using rotary evaporator and dried at room temperature. The obtained crude extracts were weighed and stored at 4^0C for the further analysis.

5.3. QUALITATIVE PHYTOCHEMICAL ANALYSIS OF AQZR [41][42][43][44]

5.3.1. Preparation of test sample

A small quantity of the extract was dissolved in 5ml of distilled water and filtered. The filtrate was tested to detect the presence of various phytochemical constituents in the sample.

5.3.2. TEST FOR CARBOHYDRATES

- **Molisch's test**

Few drops of Molisch's reagent was added to 2-3ml of filtrate, followed by addition of concentrated sulphuric acid along the sides of the test tube. Formation of violet colour ring at the junction of two liquids indicates the presence of carbohydrates.

- **Fehling's test**

1ml Fehling's-A (copper sulphate in distilled water) was added to 1ml of Fehling's-B (potassium tartarate and sodium hydroxide in distilled water) solution, boiled for one minute. To this added 1ml of filtrate and heated gently. Formation of brick red precipitate indicates the presence of reducing sugars.

- **Benedict's test**

Few ml of filtrate was mixed with equal volume of Benedict's reagent (alkaline solution containing cupric citrate complex) and heated in boiling water bath for 5min. Formation of reddish brown precipitate infers the presence of reducing sugars.

5.3.3. TEST FOR ALKALOIDS

Small amount of extract mixed with few ml of dilute hydrochloric acid. Shaken well and filtered. Following tests were performed with the obtained filtrate.

- **Dragendorff's test**

A few drops of Dragendorff's reagent (potassium bismuth iodide solution) was added to 2-3ml of filtrate. Orange red precipitate indicates the presence of alkaloids.

- **Mayer's test**

A few drops of Mayer's reagent (potassium mercuric iodide solution) was added to 2-3ml of filtrate. Cream (dull white) precipitate was formed.

- **Wagner's test**

A few drops of Wagner's reagent (solution of iodine in potassium iodide) was added to 2-3ml of filtrate. Reddish brown precipitate was obtained.

- **Hager's test**

A few drops of Hager's reagent (Picric acid) was added to 2-3ml of filtrate. Yellow precipitate was obtained.

5.3.4. TEST FOR TRITERPENOID

- **Libermann-Burchard test**

A small quantity of extract was treated with few drops of acetic anhydride, followed by a few drops of concentrated sulphuric acid. A brown ring was formed at the junction of two layers and the upper layer turns green colour, infers the presence of phytosterols and formation of deep red colour indicates the presence of triterpenoids.

- **Salkowski test**

A small quantity of the extract was treated with chloroform and few drops of concentrated sulphuric acid and allowed to stand for few minutes. Yellow colour at the lower layer indicates the presence of triterpenoids.

5.3.5. TEST FOR GLYCOSIDES

- **Legal's test**

1ml of pyridine and 1ml of sodium nitroprusside was added to 1ml of extract. Pink to red colour indicates the presence of glycosides.

- **Keller-Killiani test**

Glacial acetic acid was added to 2ml extract, followed by the addition of trace quantity of ferric chloride and 2 to 3drops of concentrated sulphuric acid. Reddish brown colour appears at the junction of two liquid indicates the presence of cardiac glycosides.

- **Baljet test:**

2ml of extract was added to sodium picrate solution. Yellow to orange colour formation indicates the presence of glycosides.

5.3.6. TEST FOR STEROIDS AND STEROLS

- **Liebermann- Burchard reaction**

2ml of extract was mixed with chloroform. To that mixture added 1-2ml of acetic anhydride and 2drops of concentrated sulphuric acid along the sides of the test tube. The solution becomes red, then blue and finally bluish green colour.

- **Salkowski reaction**

2ml of extract was mixed with 2ml chloroform and 2ml concentrated sulphuric acid. Shaken well. Chloroform layer appears red and acid layer shows greenish yellow fluorescence.

5.3.7. TEST FOR PHENOLS

- **Ferric chloride test**

1ml of the alcoholic solution of the extract was added to 2ml of distilled water followed by few drops of 10% ferric chloride. Formation of blue or green colour indicates the presence of phenols.

- **Lead acetate test**

Diluted 1ml of alcoholic solution of extract with 5ml distilled water and to this added few drops of 1% aqueous solution of lead acetate. Formation of yellow colour precipitate indicates the presence of phenols.

5.3.8. TEST FOR TANNINS

- **Lead acetate test**

A few drop of lead acetate was added to 5ml of aqueous extract. Formation of yellow or red colour precipitate indicates the presence of tannins.

5.3.9. TEST FOR SAPONINS

- **Foam Test:**

1ml of test sample was diluted with 20ml of distilled water and shaken it in a graduated cylinder for 3minutes. Foam of 1cm after 10min indicates the presence of saponins.

- **Froth test:**

5ml of test sample was added to sodium bicarbonate solution. After vigorous shaking the mixture, kept it for 3minutes. A honey comb like froth formation indicates the presence of saponins.

5.3.10. TEST FOR FLAVONOIDS

- **Alkaline reagent test**

A few drop of sodium hydroxide solution was added to the extract. Formation of an intense yellow colour, which turns to colourless on addition of few drops of dilute hydrochloric acid, indicates the presence of flavonoids.

- **Shinodas test [Magnesium hydrochloride reduction test**]

Alcoholic solution of extract was treated with a small piece of magnesium ribbon and a few drops of concentrated HCl was added and heated. Appearance of crimson red or occasionally green to blue colour infers the presence of flavonoid.

5.3.11 TEST FOR PROTEINS AND AMINO ACIDS

- **Biuret test**

3ml of test solution was added to 4% sodium hydroxide and few drops of 1% copper sulphate solution. Formation of violet colour indicates the presence of proteins.

- **Ninhydrin test**

A mixture of 3ml test solution and 3drops of 5% Ninhydrin solution was heated in a boiling water bath for 10min. Formation of purple or bluish colour indicates the presence of free amino acids.

5.4. QUANTIFICATION OF TOTAL PHENOLICS AND FLAVONOIDS [45][46]

5.4.1. ESTIMATION OF TOTAL PHENOLICS

Reagents

- Folin-Ciocalteu's reagent
- Gallic acid (1mg/ml)
- 20% sodium carbonate

Preparation of standard

Standard solution of was prepared by adding 10mg of accurately weighed Gallic acid in 10ml of distilled water.

Preparation of sample

10mg of the accurately weighed AQZR extract were separately dissolved in 10ml ethanol and used for the estimation.

.**Procedure**

The total phenolic content of the AQZR were determined by Folin-Ciocalteu assay method. To an aliquot 100µl of AQZR (1mg/ml) or standard solution of Gallic acid (10, 20, 40, 60, 80, 100µg/ml) added 50µl of Folin-ciocalteu reagent followed by 860µl of distilled water and the mixture is incubated for 5min at room temperature. 100µl of 20% sodium carbonate and 890µl of distilled water were added to make the final solution to 2ml. It was incubated for 30min in dark to complete the reaction. After that absorbance of the mixture was measured at 725nm against blank. Distilled water was used as reagent blank. The tests were performed in triplicate to get mean the values. The total phenolic content was found out from the calibration curve of Gallic acid. And it was expressed as milligrams of Gallic acid equivalents (GAE) per gram of extract.

5.4.2. ESTIMATION OF TOTAL FLAVONOIDS

Reagents

- Ethanol
- 10% Aluminium chloride
- 1M Potassium acetate

Preparation of standard

Standard solution of was prepared by adding 10mg of accurately weighed Quercetin in 10ml of ethanol.

Preparation of sample

10mg of the accurately weighed AQZR extracts were separately dissolved in 10ml ethanol and used for the estimation.

Procedure

The total flavonoid content of the AQZR was determined by using Aluminium chloride colorimetric method. To an aliquot of 100µl of extract (1mg/ml) or standard solutions of Quercetin (10, 20, 40, 60, 80, 100µg/ml) methanol was added separately to make up the solution upto 2ml. The resulting mixture was treated with 0.1ml of 10% aluminium chloride, 0.1ml of 1M potassium acetate and 2.8ml of distilled water. Shaken well and incubated at room temperature for 30minutes. The absorbance was measured at 415nm against blank, where a solution of 2ml ethanol, 0.1ml potassium acetate, 2.8ml distilled water and 0.1ml of aluminium chloride serve as blank solution. The total flavonoid content was determined from the standard Quercetin calibration curve. And it was expressed as milligrams of Quercetin equivalents (QE) per gram of extract.

5.5. *INVITRO* ANTIOXIDANT STUDY OF AQZR

5.5.1. DPPH Free Radical Scavenging Assay [47]

Principle

The DPPH [1, 1-diphenyl-2-picrylhydrazyl (α, α-diphenyl-β-picrylhydrazyl)] assay method is based on the reduction of DPPH, a stable free radical. The free radical DPPH with an odd electron gives a maximum absorption at 517nm (purple colour). When Antioxidants react with DPPH, which is a stable free radical becomes paired off in the presence of a hydrogen donor (e.g., a free radical scavenging antioxidant) and is

reduced to the DPPHH and as consequence the absorbance's decreased from the DPPH. DPPH radical is a stable radical by virtue of the delocalization of the spare electron over the molecule as a whole, so that the molecules do not dimerize, as would be the case with most other free radicals. The delocalization also gives rise to the deep violet colour, characterized by an absorption band in ethanol/methanol solution centred at about 520nm Radical to the DPPH-H form, results in decolorization (yellow colour) with respect to the number of electrons captured. More the decolorization more is the reducing ability. This test has been the most accepted model for evaluating the free radical scavenging activity of any new drug. When a solution of DPPH is mixed with that of a substance that can donate a hydrogen atom, then this gives rise to the reduced form (Diphenyl picryl hydrazine; non radical) with the loss of this violet colour (although there would be expected to be a residual pale yellow colour from the picryl group still present).

Procedure

The method adopted here is Blois method. Where by using the stable DPPH radical, the antioxidant capacity of the extract was measured in terms of hydrogen donating or radical scavenging ability. 1ml of 0.3mM solution of DPPH in ethanol was added to various concentrations of sample (10, 20, 40, 60, 80, 100 µg/ml) and the reference compound (5, 10, 15, 20, 25 and 30 µg/ml), shaken vigorously, and left to stand in the dark at room temperature. After 30 min absorbance was measured at 517nm against a blank. Quercetin was used as reference compound. A control reaction was also carried out without the test sample. All the tests were performed in triplicate in order to get the mean values. The percentage of inhibition was calculated by comparing the absorbance values of the control and test samples. Free radical scavenging activity was expressed as percentage inhibition (I%) and calculated using the following equation:

Percentage inhibition (I%) = (Abs $_{control}$- Abs $_{sample}$ /Abs $_{control}$) X 100

Different sample concentrations were used in order to obtain calibration curves and to calculate the IC_{50} values. (IC_{50} - concentration required to obtain a 50% radical scavenging activity).

5.5.2. ABTS Assay [48][49]

Principle

A method for the screening of antioxidant activity is reported as a decolorization assay applicable to both lipophilic and hydrophilic antioxidants, including flavonoids, hydroxyl cinnamates, carotenoids, and plasma antioxidants. The pre-formed radical monocation of 2,2'-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS*+) is generated by oxidation of ABTS with potassium persulfate and is reduced in the presence of such hydrogen-donating antioxidants present in the sample. The influences of both the concentration of antioxidant and duration of reaction on the inhibition of the radical cation absorption are taken into account when determining the antioxidant activity. Chemistry involves the direct generation of the ABTS radical monocation with no involvement of an intermediary radical. It is a decolorization assay; thus the radical cation is pre-formed prior to addition of antioxidant test systems, rather than the generation of the radical taking place continually in the presence of the antioxidant. An antioxidant with an ability to donate a hydrogen atom will quench the stable free radical and inhibits the absorption of the radical cation which has characteristic long-wavelength absorption spectrum showing maxima at 660,734, and 820nm.The relatively stable ABTS radical has a green colour and is quantified spectrometrically at 734nm. It is applicable to both aqueous and lipophilic systems.

Procedure

ABTS radical scavenging activity of the extract was measured by Rice-Evans method. ABTS was dissolved in water to a 7mM concentration. ABTS radical cation ($ABTS^{+}$) was produced by reacting ABTS stock solution with 2.45mM potassium persulfate and allowing the mixture to stand in the dark at room temperature for 12- 16hr before use. The radical was stable in this form for more than 2 days when stored in the dark at room temperature. For the study, ABTS solution was diluted with phosphate buffer saline pH 7.4 (PBS) to an absorbance of 0.70 (± 0.02) at 734nm and equilibrated at 30^{0}C. After addition of 1ml of diluted ABTS solution to various concentrations of sample or reference compound (Quercetin), the reaction mixture was incubated for 6min and then absorbance was measured at 734nm against a blank. A control reaction was carried out without the sample. All the tests were performed in triplicate in order to get

the mean values. The percentage inhibition of $ABTS^{+}$ by the sample was calculated according to the formula:

Percentage inhibition (I %) = (Abs $_{control}$- Abs $_{sample}$ /Abs $_{control}$) X 100

Different sample concentrations were used in order to obtain calibration curves and to calculate the EC_{50} values. (EC_{50} - concentration required to obtain a 50% radical scavenging activity).

5.5.3. Nitric Oxide Assay [50][51]

Principle :

Nitric oxide (NO) and reactive nitrogen species (RNS) are free radicals are derived from the interaction of NO with oxygen or reactive oxygen species. Nitric oxide is classified as a free radical because of its unpaired electron and displays important reactively with certain types of proteins and other free radical such as superoxide. NO is synthesized by three isoforms of the enzymatic nitric oxide synthase (NOS), endothelial NOS, neuronal NOS, and inducible NOS (iNOS). Nitric oxide (NO) is generated from amino acid L-arginine by the enzymes in the vascular endothelial cells, certain neuronal cells, and phagocytes. Low concentration of NO are sufficient in most cases to affect the physiological function of the radical. NO is a diffusible free radical that plays many roles as an effectors molecule in diverse biological systems including neuronal messenger, vasodilation and antimicrobial and antitumor activities. Chronic exposure to nitric oxide radical is associated with various carcinomas and inflammatory conditions including juvenile diabetes, multiple sclerosis, arthritis and ulcerative colitis. The toxicity of NO increases greatly when it react with the superoxide radical, forming the highly reactive peroxy nitrite anion ($ONOO^{-}$). Nitric oxide has been shown to be directly scavenged by flavonoids.

Procedure:

The nitric oxide scavenging activity was conducted based on the Greiss assay method. 2.0 mL of 10 mM sodium nitroprusside and 0.5mL of phosphate buffer were mixed with 0.5mL of different concentration of the plant extract and incubated at 25ºC

for 150 min. The sample was run as above but the blank was replaced with the same amount of water. After the incubation period, 2ml of the above incubated solution was added to 2ml of Griess reagent and incubated at room temperature for a period of 30mins. The absorbance of the chromophore formed was read at 540nm.

Percentage inhibition (I%) = (Abs $_{control}$- Abs $_{sample}$ /Abs $_{control}$) X 100

5.6 ANTI-MICROBIAL ACTIVITY OF AQZR [52]

5.6.1. Antibacterial study of AQZR

Preparation of inoculums

The inoculums for the experiment were prepared in fresh nutrient broth from the preserved slant culture. The turbidity of the culture can be adjusted by the addition of broth or sterile saline (if it is excessive) or by further incubation to get the required turbidity, and the newly prepared inoculums were standardized by adjusting the turbidity of the culture to that of McFarland standards.

Preparation of sterile swabs

Cotton wool swab on wooden applicator or plastics were prepared and sterilized by autoclaving or by dry heat (only for the wooden swabs). It was sterilized by packing the swabs in culture tubes, papers or tins etc.

Sterilization of forceps

Forceps can be sterilized by dipping in alcohol and burning off the alcohol.

Experiment

The standardized inoculums is inoculated in the sterilized plates prepared earlier (aseptically) by dipping a sterile in the inoculums removing the excess of inoculums by passing and rotating the swab firmly against the side of the culture tube above the level of the liquid and finally streaking the swab all over the surface of the medium 3times rotating the plate through an angle of 60º after each application. Finally pass the swab round the edge of the agar surface. Leave the inoculums to dry at room temperature with the lid closed. The sterile discs are soaked overnight in sample solutions, AQZR. Each

petri dish is divided into 2 parts. First compartment were loaded with sample disc as AQZR (100µg) and second compartment with standard Ciprofloxacin disc (10µg) with the help of sterile forceps. After that petri dishes are placed in the refrigerator at 4º C or at room temperature for 1hour for diffusion. Incubate at 37ºC for 24hours. Observe the zone of inhibition produced by different samples. Measure it using a scale and record the average of two diameters of each zone of inhibition.

Table 2: Bacterial strain used for the study with NCIM

Sl no	Organism	Strain	NCIM
1	**Gram+ve bacteria**	*Bacillus subtilis*	*2063*
2		*Staphylococcus aureus*	*2079*
3	**Gram–ve bacteria**	*E-coli*	*2065*
4		*Pseudomonas auregenosa*	2200

5.6.2. Anti fungal activity of AQZR

Procedure

Preparation of inoculums

The inoculums for this particular experiment were prepared in fresh sabouraud dextrose broth from preserved slant culture. The turbidity of the culture can be adjusted by the addition of broth or sterile saline (if it is excessive) or by further incubation to get the required turbidity, and the newly prepared inoculums were standardized by adjusting the turbidity of the culture to that of McFarland standards.

Preparation of sterile swabs

Cotton wool swab on wooden applicator or plastics were prepared and sterilized by autoclaving or by dry heat (only for the wooden swabs). It was sterilized by packing the swabs in culture tubes, papers or tins etc.

Sterilization of forceps

Forceps can be sterilized by dipping in alcohol and burning off the alcohol.

Experiment

The standardized inoculums is inoculated in the sterilized plates prepared earlier (aseptically) by dipping a sterile in the inoculums removing the excess of inoculums by passing and rotating the swab firmly against the side of the culture tube above the level of the liquid and finally streaking the swab all over the surface of the medium 3times rotating the plate through an angle of 60º after each application. Finally pass the swab round the edge of the agar surface. Leave the inoculums to dry at room temperature with the lid closed. The sterile discs are soaked overnight in sample solutions, AQZR. Each Petri dish is divided into 2 parts. First compartment were loaded with sample disc as AQZR (100µg) and second compartment with standard Fluconazole disc (10µg) with the help of sterile forceps. After that petri dishes are placed in the refrigerator at 4ºC or at room temperature for 1hour for diffusion. Incubate at 37ºC for 24hours. Observe the zone of inhibition produced by different samples. Measure it using a scale and record the average of two diameters of each zone of inhibition.

Table 3: Fungal strain used for the study with MTCC

Si no	Fungi strains used	MTCC
1	***Aspergillus niger***	1344
2	***Monascus purpureus***	1090

5.6.3. DETERMINATION OF MINIMUM INHIBITORY CONCENTRATION OF AQZR [53][54]

The minimum inhibitory concentration (MIC) is the lowest concentration of a chemical that prevents the visible growth of the organism.ie, the lowest concentration at which it has bacteriostatic activity.

5.6.3.1. Preparation of test drug:

The selected test drugs were prepared in DMSO at a concentration 2000µg/ml

5.6.3.2. Preparation of inoculums:

Bacillus subtilis, *Staphylococcus aureus*, *E-coli*, *Pseudomonas auregenosa*, *Aspergillus niger*, and *Monascus purpureus* were the strains of organisms selected for the study. Overnight culture are grown at 37^0C Kirby- Bauer procedure and diluted to Muller Hinton Broth and Sabouraud Dextrose Broth for bacterial and fungal strains respectively. This overnight culture was diluted to 10^{-2}.

Inoculation

1. The sterile tubes were labeled 1-8 and 8^{th} tube was taken as control.
2. 1ml of Muller Hinton Broth was transferred to all tube.
3. 1ml of drug solution was added to 1^{st} tube and mixed well.
4. From the 1^{st} tube transfer 1ml of solution to the 2^{nd} tube and was repeated up to 7^{th} tube.
5. From the 7^{th} tube 1ml of solution was pipette out and discarded.
6. 0.01ml of culture was added to all the test tubes.
7. All the tubes were incubated at 37^0C for 18-24hrs.
8. After incubation observe the turbidity by visually
9. The highest dilution without growth is the minimal inhibitory concentration.

5.7. .PHARMACOLOGICAL EVALUATION OF AQZR

5.7.1. ACUTE TOXICITY STUDY [55]

Based on previously conducted study the dose was selected.

5.7.2. ANIMALS AND MANAGEMENT

Female Wistar rats of 6-8 weeks old and 160-180g body weight were offered by KMCH College of Pharmacy, Coimbatore. All rats were kept at room temperature and allowed to accommodate in standard conditions at 12-hr light and 12-hr dark cycle in the animal house. Animals were fed with commercial pellet diet and water *ad libitum* freely throughout the study. The experimental procedure was approved by IAEC (Institutional animal ethical committee of KMCH, governed by CPCSEA, Government of India.

5.7.3. DRUG

Commercially available Povidone Iodine Ointment (5%) were used as standard drug. The ointments were applied topically over the wound area.

5.7.4. PREPARATION OF TEST DRUG

AQZR ointment was formulated using (1%) AQZR stem extract in simple base ointment.

5.7.5. PREPARATION OF SIMPLE OINTMENT BASE [56]

Ingredients:

Wool fat	5.0g
Hard paraffin	5.0g
Cetostearyl alcohol	5.0g
White soft paraffin	85.0g

Type of preparation: Absorption ointment base

Procedure:

Hard paraffin and cetostearyl alcohol taken in a china dish kept on water-bath at 70^0C. Wool fat and white soft paraffin are added to this mixture and stirred until all the ingredients are melted. If required decanted or strained and stirred until cold and packed in suitable container.

1% of AQZR were separately mixed with the above prepared simple ointment base.

5.7.6. EXPERIMENTAL MODEL

5.7.7. EVALUATION OF WOUND HEALING EFFECT OF AQZR IN EXCISION WOUND MODEL

Rats were divided into 4 groups each containing 5 animals as follows.

Table 4: Experimental design for excision wound model

GROUP	GROUP SPECIFICATION	INTERVENTION
Group I	Control	Untreated
Group II	Simple base ointment	Only with simple base ointment
Group III	Standard	Povidone iodine ointment 5%
Group IV	Test 1	AQZR ointment 1%

INDUCTION OF WOUND [57]

On wounding day the rats were anaesthetized prior to creation of the wounds, by ether anaesthesia. The dorsal fur of the animal was shaved with an electric clipper and the area of the wound to be created was outlined on the back of the animals with methylene blue using a circular stainless steel stencil. A full thickness of the excision wound of 1.5cm in width (circular area 2.25cm^2) created along the markings using toothed forceps, a surgical blade and pointed scissors. The entire wound left open. All the surgical interventions were carried out under sterile condition. After 24h of wound creation, the ointments was applied gently to cover the wounded area once daily until complete healing Wound area and wound contraction, epithelializtion period and hydroxyproline content were monitored.

5.8. ESTIMATION OF PARAMETERS

5.8.1. Measurement of wound contraction [58]

The progression of wound healing was judged by the periodic assessment of the contraction of excision wounds. Wound contraction was monitored by tracing the outline of the wound on tracing sheet and then using graph sheet to calculate the area of the wound size. All animals in each group were monitored until complete healing of wounds occurred and the day at which each wound healed was recorded. Mean of all healed wounds was determined.

$$\text{Percent wound contraction} = \frac{\text{Healed area}}{\text{Total area}} \times 100$$

5.8.2. Estimation of hydroxyproline [59][60]

Principle

The procedure is based on alkaline hydrolysis of the tissue homogenate and subsequent determination of the free hydroxyproline for the production of a pyrole. The addition of Ehrlich's reagent resulted in the formation of achromophore that can be measured at 557nm. Optimal assay conditions were determined using tissue homogenate and purified acid soluble collagen along with standard hydroxyproline. Critical parameters such as the amount of chloramine-T, sodium hydroxide, p-dimethylaminobenzaldehyde, pH of the reaction buffer, and length of oxidation time were observed to obtain satisfactory results.

Procedure

Known amount of tissue (50mg) was taken in glass tubes and 4ml of 6N HCl was added to each tube to hydrolyse the tissue sample. The glass tubes were sealed and were incubated for 22 hours. The tubes are then opened and the contents are decanted into a china dish. HCl was then removed by evaporation and the residue was dissolved in water and made up to known volume (10ml) using a standard flask. A series of standards were prepared containing 20-200µg of hydroxyproline with a final volume of 2ml. 1ml of the hydrolyzed tissue samples was used to estimate the contents of hydroxyproline. Hydroxyproline oxidation was initiated by adding 1ml of chloramine-T to each tube in a predetermined sequence. The tube content were mixed by shaking a few minutes and

allowed to stand for 20min at room temperature. Chloramine-T was then destroyed by adding 1ml of perchloric acid to each tube in the same order as before. The contents were mixed and allowed to stand for 5min. Finally 1ml of p-Dimethyl aminobenzaldehyde solution was added and the mixture was shaken well. Tubes were placed in a 60°C water bath for 20min and then cooled in tap water for 5min. The colour developed was read spectrophotometrically at 557nm. Hydroxyproline value was determined from the standard curve.

5.8.3 Estimation of Hexosamine [59] [61]

Principle

The method was based on the observation that in alkaline solution at 100°C, the amino sugars react with acetyl acetone to form chromogenic material which gives a chromophore or chromophores on treatment in acid solution with ethanolic p-dimethyl amino benzaldehyde. The method described is suitable for the estimation only for free amino sugars where a determination is carried to as certain the amino sugar content of a polysaccharide or other material of high molecular weight. Any amino sugar units remaining as oligosaccharide or substituted aminosugar gives less color per unit weight of amino sugar than that found for free amino sugar. Therefore it is essential that the hydrolysis of granulation tissue is done without destruction of the amino sugar.

Procedure

Tissue samples (50mg) were hydrolyzed with 2NHCL (5ml) at 100°C for 6hrs. Hydrochloric acid was then removed by evaporation, then the residue was dissolved in water and made up to a known volume (10ml) using a standard flask. Aliquots containing 10-50mg hexosamine were treated with 1ml of freshly prepared 2% acetyl acetone in 0.5M sodium carbonate in capped tubes and kept in boiling water bath for 15min. After cooling in tap water, 5ml of 95% ethanol and 1ml of Ehrlich's reagent (1.33%Dimethyl amino benzaldehyde in 1:1 ethanol: concentrated hydrochloric acid mixture) were added and mixed thoroughly. The purple red colour developed was read after 30min at 530 nm. Water blank and standard glucosamine solution of various concentrations were also treated similarly to get a standard curve.

5.8.4. Estimation of Uronic acid [59][62]

Principle

The galacturonic acid content of the hydrolyzed sample is quantified colorimetrically using a modification of the Copper reduction procedure originally described by Avigad and Milner. This modification, substituting the commonly used Folin-Ciocalteu reagent for the arsenic containing Nelson reagent, gives a response that is linear, sensitive, and selective for uronic acids over neutral sugars. This method also avoids the use of concentrated acids needed for the commonly used m-phenyl phenol method. This combined enzymatic and colorimetric procedure correctly determined the galacturonic acid and methanol content of purified sample. In both cases good agreement was obtained between this method and commonly used methods.

Procedure

A buffered copper solution was prepared by adding 23.2g NaCl, 3.2g sodium acetate, and 1ml glacial acetic acid to 80ml water. Once dissolved, 0.5g CuSO4 is added, the pH adjusted to 4.8 with NaOH, and the final volume brought to 100ml. This solution is stable for weeks at room temperature. For the assay, equal volumes of this solution and the sample are mixed, giving final reagent concentrations in the assay of 2M NaCl, 0.2M acetate, and 10mM CuSO4. In our standard assay, sample and assay solution volumes of 0.1ml each are mixed in test tubes, then the tubes are covered with glass marbles and placed in an aluminum heating block at 100 C. A diluted Folin–Ciocalteu reagent is then prepared by mixing 1ml of 2N Folin–Ciocalteu with 39ml of water. After 40min, the samples are removed from the heat and 8 volumes (0.8ml in our standard assay) of the 40-fold diluted Folin–Ciocalteu reagent is added. A colored product forms immediately; absorbance was measured at 750nm. Where the BCA reagent was used the procedure was the same, except instead of adding the diluted Folin– Ciocalteu reagent, 0.8mL of solution ‘‘A” from the procedure described in Waffenschmidt and Jaenicke, containing 5.0mM BCA in pH 10.1 carbonate buffer, was added. Absorbance was measured at 560nm.

5.9. HISTOLOGICAL ASSESSMENT [59]

Histological studies of wounded tissues provide accurate diagnosis of level of healing of the wound. Histopathology is the microscopical study of tissues for pathological alterations. This involves collection of morbid tissues from biopsy or necropsy, fixation, preparation of sections, staining and microscopical examination.

Collection of materials

Thin pieces of 3 to 5mm, thickness were collected from tissues showing gross morbid changes along with normal tissue.

Fixation:

Kept the tissue in fixative for 24-48hours at room temperature

The fixation was useful in the following ways:

a) Serves to harden the tissues by coagulating the cell protein,
b) Prevents autolysis,
c) Preserves the structure of the tissue, and
d) Prevents shrinkage

Common Fixatives: 10% Formalin

5.10. STATISTICAL ANALYSIS

Data's of all the parameters were analyzed using the Graph pad 5.0 software. Analysis of Variance (ANOVA); one way ANOVA followed by Dunnets comparison test was performed. The values were expressed as Mean ± SEM. P value<0.05 was considered as significant.

6. RESULTS

6.1 EXTRACTIVE YIELD OF AQUEOUS STEM EXTRACT OF *Zanthoxylum rhetsa* (Roxb.) DC

Percentage Yield:

Coarsely powdered *Zanthoxylum rhetsa* (Roxb) DC stem were extracted with aqueous using maceration technique.

The percentage yield of Aqueous stem extract of *Zanthoxylum rhetsa* (Roxb)DC (AQZR) was found to be 14 % w/w.

6.2 PRELIMINARY PHYTOCHEMICAL ANALYSIS OF AQZR

Table 5: Phytochemical Analysis of *Zanthoxylum rhetsa* (Roxb) DC

S.NO	PHYTOCHEMICAL CONSTITUENTS	AQZR
1	Alkaloids	Positive
2	Proteins and Aminoacids	Positive
3	Flavonoids	Positive
4	Phenols	Positive
5	Glycosides	Positive
6	Tannins	Positive
7	Carbohydrates	Positive

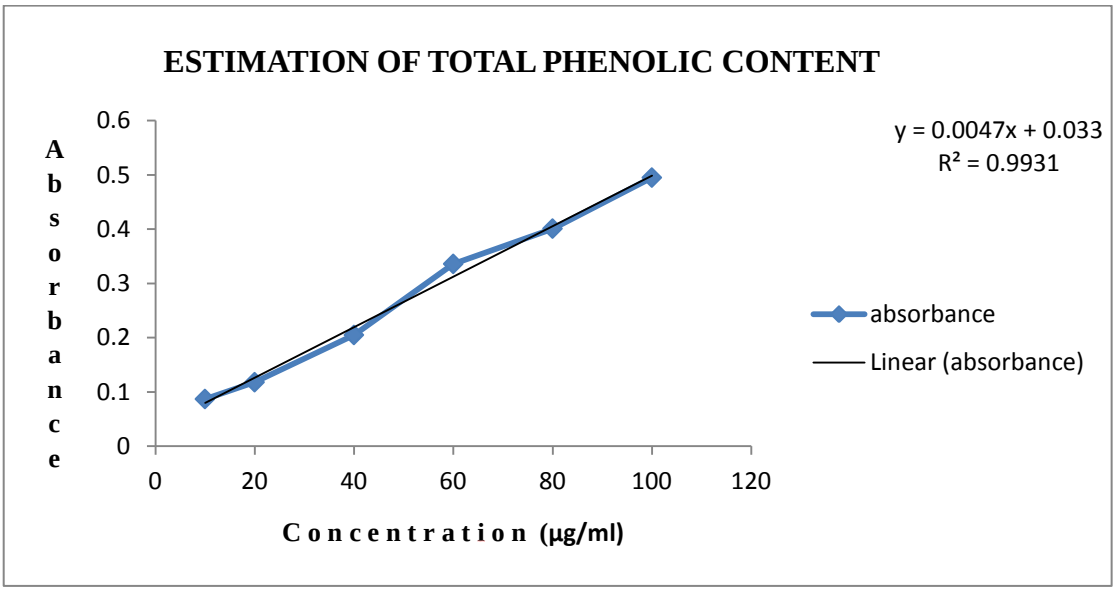
6.3 QUANTIFICATION OF TOTAL PHENOL AND FLAVONOIDS

6.3.1 ESTIMATION OF TOTAL PHENOL OF AQZR

Table 6: Estimation of total phenolic content of AQZR

Sample	Concentration (µg/ml)	Absorbance
Standard (Gallic acid) 1mg/ml	10	0.087
	20	0.118
	40	0.205
	60	0.336
	80	0.401
	100	0.495
AQZR	100	0.3185

Figure 2: Estimation of total phenolic content of AQZR



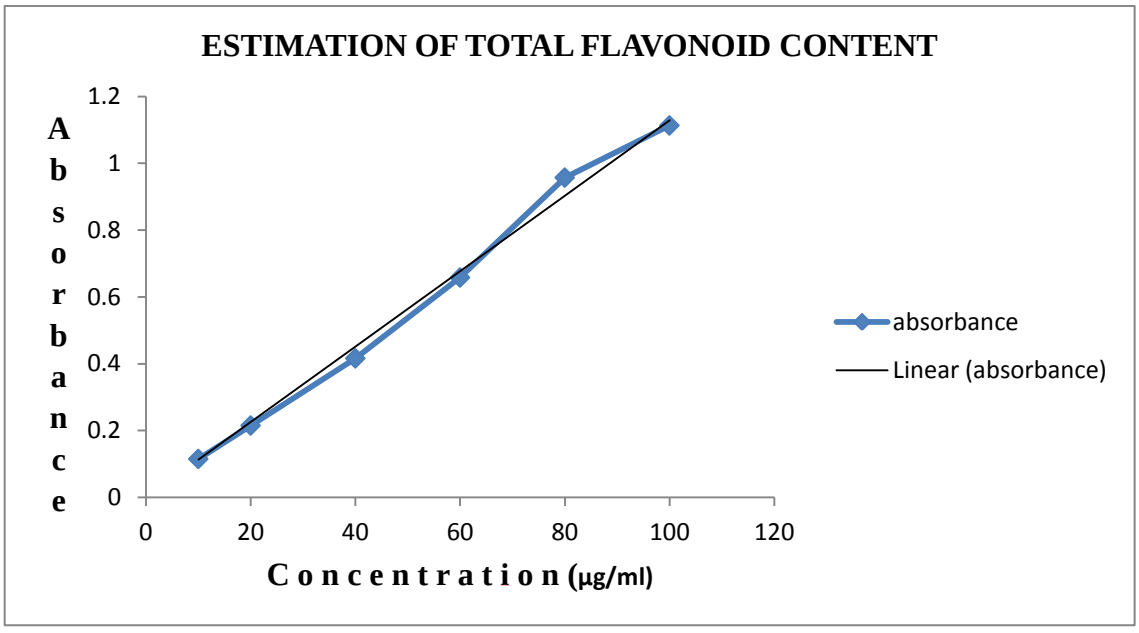
The total phenolic content in AQZR was found to be 71.37mg/g of extract calculated as Gallic acid equivalent.

6.3.2 ESTIMATION OF TOTAL FLAVONOID CONTENT OF AQZR

Table 7: Estimation of total flavonoid content of AQZR

Sample	Concentration (µg/ml)	Absorbance
Standard (Quercetin) 1mg/ml	10	0.031
	20	0.085
	40	0.26
	60	0.5026
	80	0.776
	100	1.053
AQZR	100	0.7046

Figure 3: Estimation of total flavonoid content of AQZR



The total flavonoid content in AQZR was found to be 40.64 mg/g of extract calculated as Quercetin equivalent.

6.5 *IN VITRO* ANTIOXIDANT ACTIVITY OF AQZR

6.5.1 DPPH RADICAL SCAVENGING ACTIVITY

Table 8: Percentage inhibition and IC_{50} values of DPPH radical by Quercetin

Sl.No	Concentration (µg/ml)	Percentage inhibition	IC_{50}(µg/ml)
1	5	41.2	1.55
2	10	55.7	
3	15	69.4	
4	20	86.4	
5	25	96.5	
6	30	99.38	

Fig 4: DPPH radical scavenging activity of Quercetin

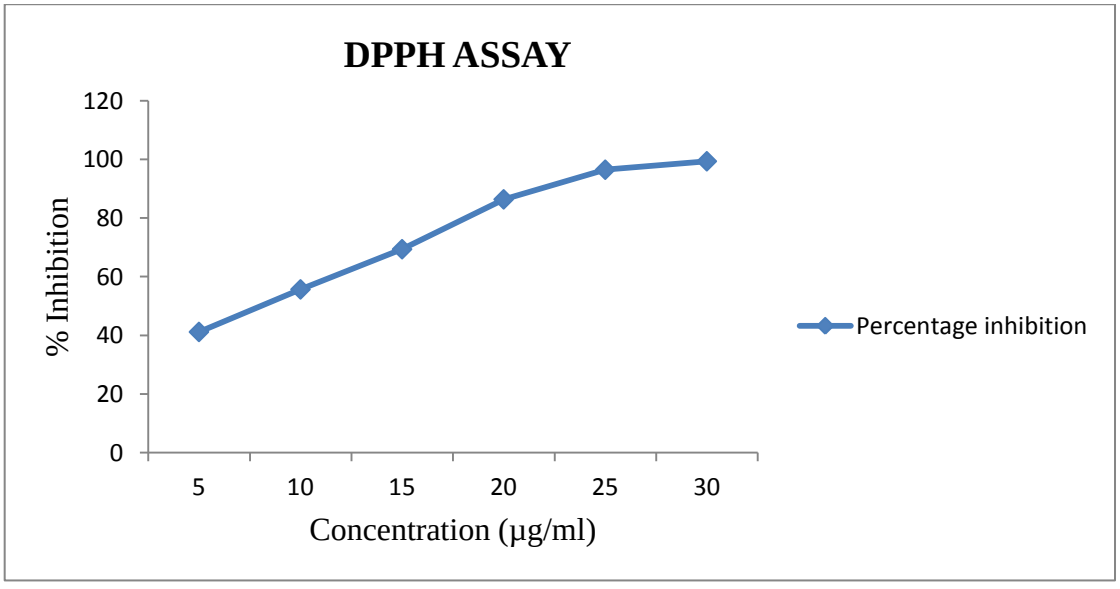
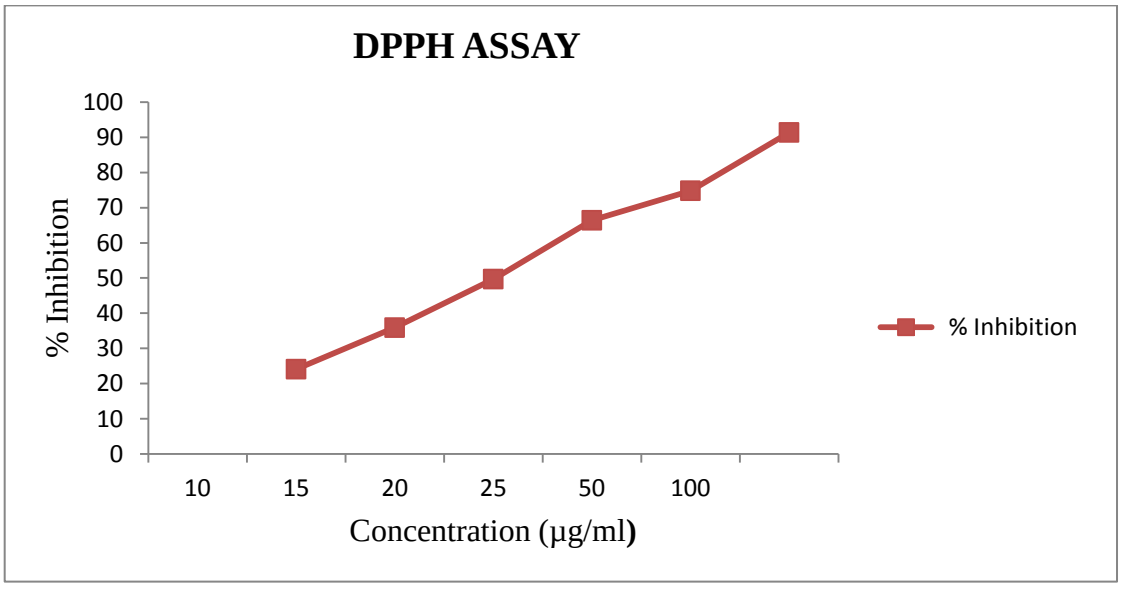


Table 9: Percentage inhibition and IC_{50} values of DPPH radical by AQZR

Sl.No	Concentration (µg/ml)	Percentage inhibition	IC $_{50}$(µg/ml)
1	10	26.69	17.77
2	15	43.89	
3	20	50.12	
4	25	59.67	
5	50	69.77	
6	100	91.36	

Fig 5: DPPH radical scavenging activity of AQZR



6.5.2 TOTAL ANTIOXIDANT ACTIVITY BY ABTS RADICAL CATION ASSAY

Table 10: Percentage inhibition and IC_{50} values of ABTS radical by Quercetin

Sl.No	Concentration (µg/ml)	Percentage inhibition	IC_{50}(µg/ml)
1	0.25	70.08	0.1142
2	0.5	75.22	
3	0.75	79.62	
4	1.0	85.88	
5	1.25	91.9	
6	1.75	98.85	

Fig 6: ABTS radical scavenging activity of Quercetin

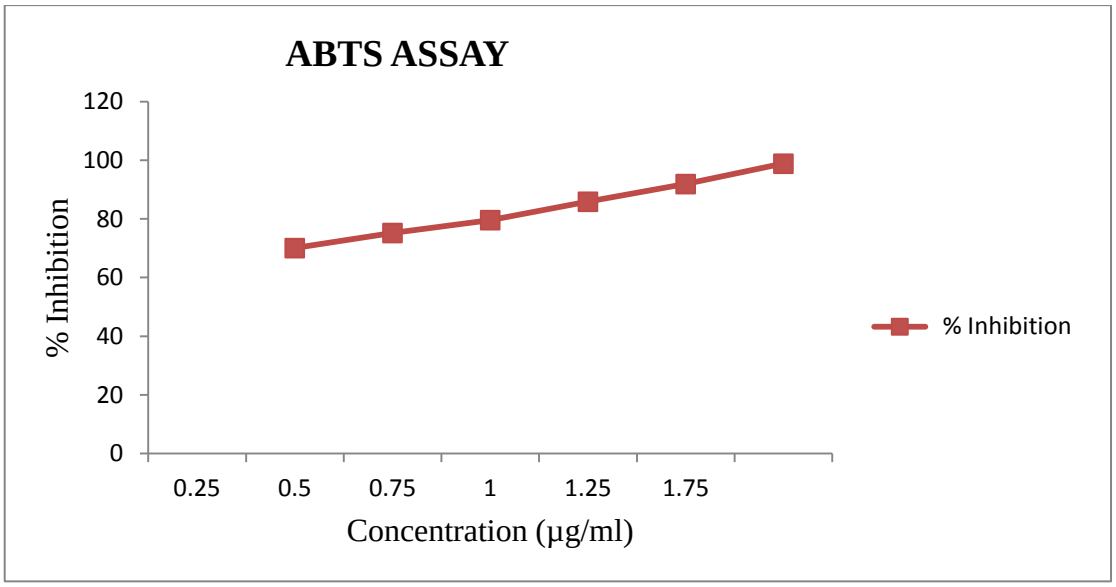
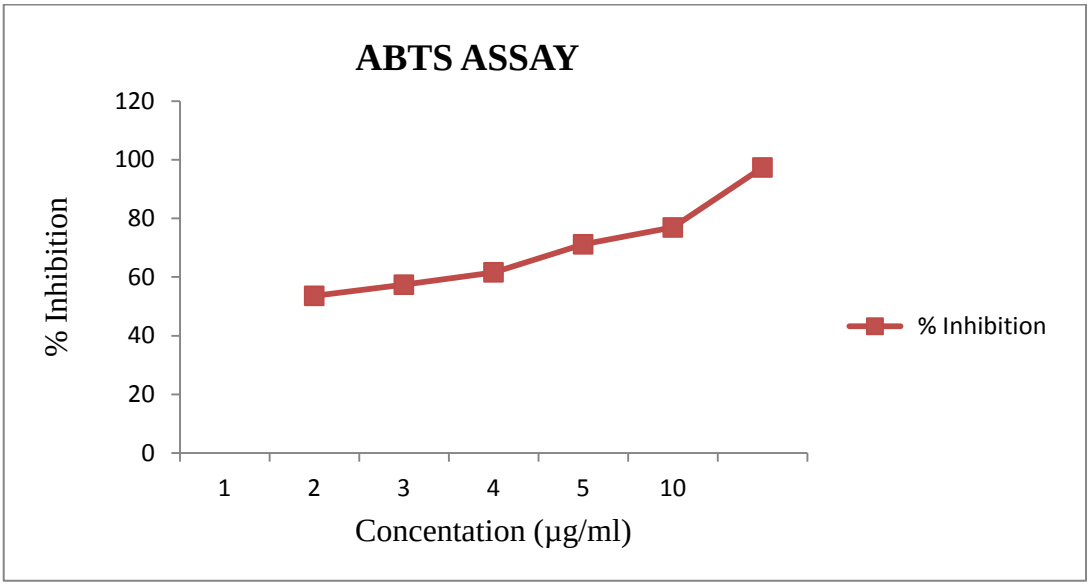


Table 11: Percentage inhibition and IC_{50} values of ABTS radical by AQZR

Sl.No	Concentration (µg/ml)	Percentage inhibition	IC_{50} (µg/ml)
1	1	53.61	1.160
2	2	57.41	
3	3	61.63	
4	4	71.18	
5	5	76.92	
6	10	97.37	

Fig 7: ABTS radical scavenging activity of AQZR



6.5.3 NITRIC OXIDE RADICAL SCAVENGING ACTIVITY

Table 12: Percentage inhibition and IC_{50} values of NO radical by Quercetin

Sl.No	Concentration (µg/ml)	Percentage inhibition	IC_{50}(µg/ml)
1	1	41.2	1.55
2	2	55.7	
3	3	69.4	
4	4	86.4	
5	5	96.5	

Fig 8: NO radical scavenging activity of Quercetin

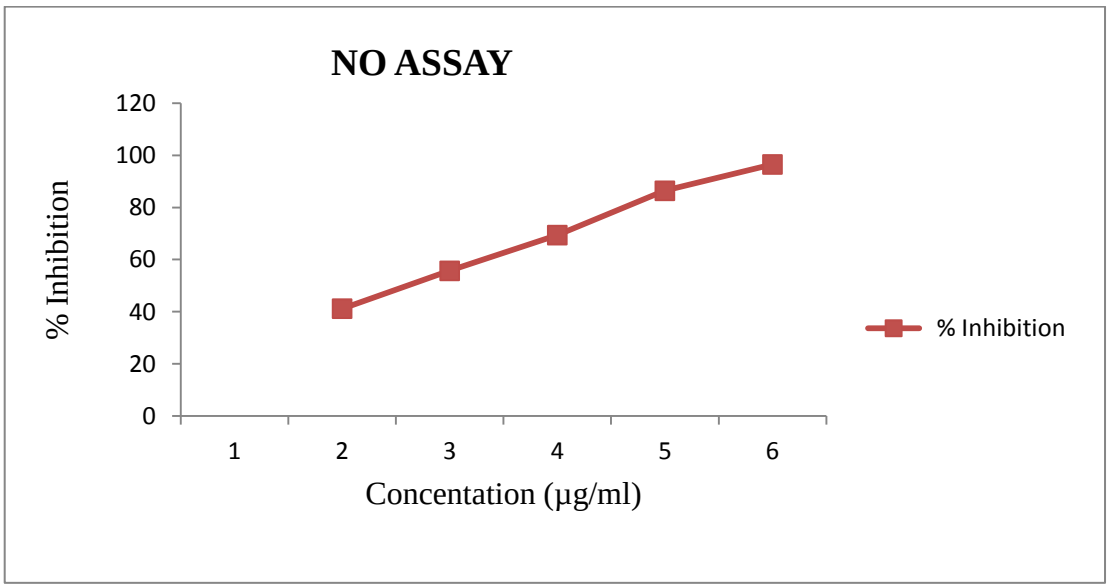
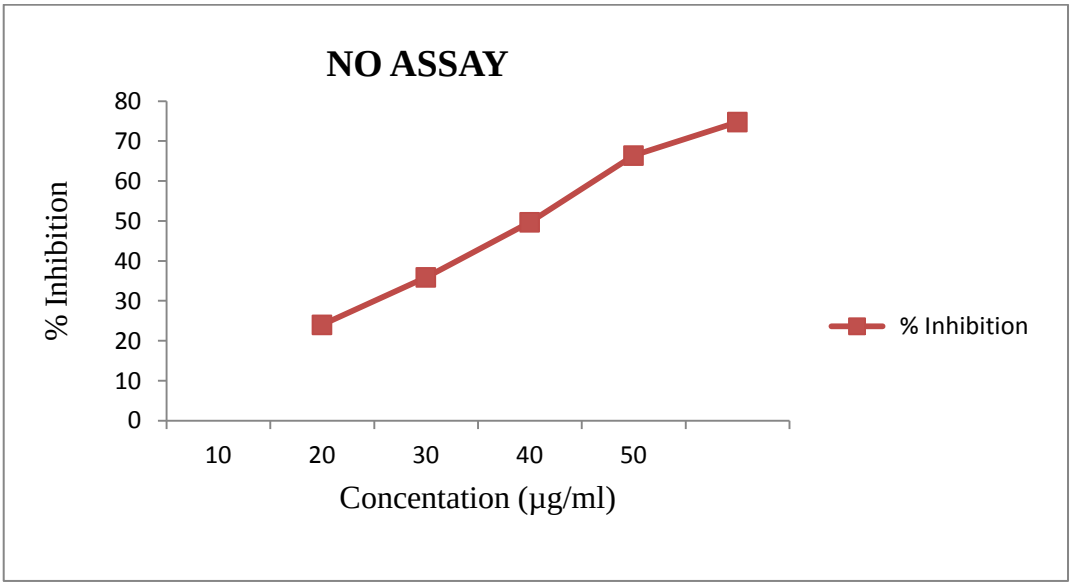


Table 13: Percentage inhibition and IC_{50} values of NO radical by AQZR

Sl.No	Concentration (µg/ml)	Percentage inhibition	IC_{50} (µg/ml)
1	10	24	30.5
2	20	35.9	
3	30	49,7	
4	40	66.4	
5	50	74.8	

Fig 9: NO radical scavenging activity of AQZR



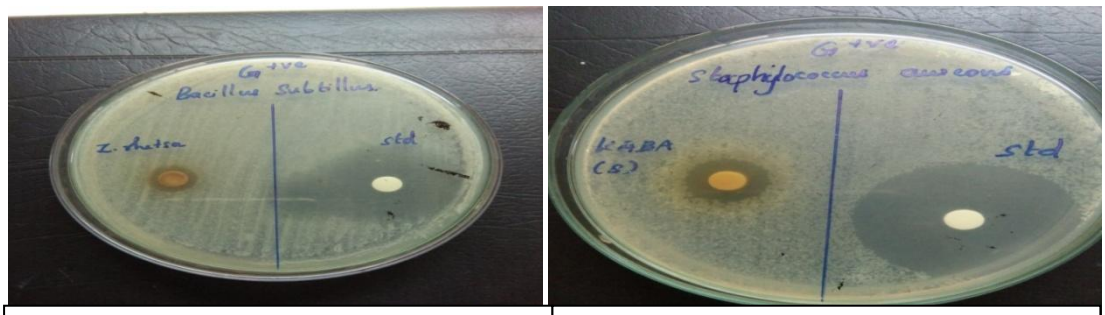
6.6 ANTI MICROBIAL ACTIVITY OF AQZR

ANTI BACTERIAL ACTIVITY

Table 14: Zone of inhibition for Gram +ve organisms

Sino	Gram +ve organisms	Standard Ciprofloxacin(mm)	AQZR(mm)
1	*Bacillus subtilis*	25	10
2	*Staphylococcus aureus*	26	14

Figure 10: Zone of inhibition for Gram +ve organisms

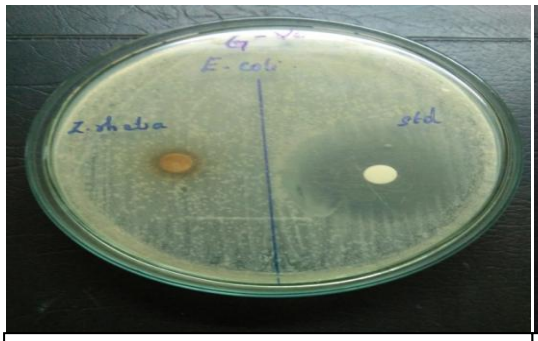


Bacillus subtilis	***Staphylococcus aureus***

Table 15: Zone of inhibition for Gram –ve organisms

Sino	Gram –ve organisms	Standard Ciprofloxacin(mm)	AQZR(mm)
1	*E.coli*	26	9
2	*Pseudomonas auregenosa*	27	10

Figure 11: Zone of inhibition for Gram –ve organisms



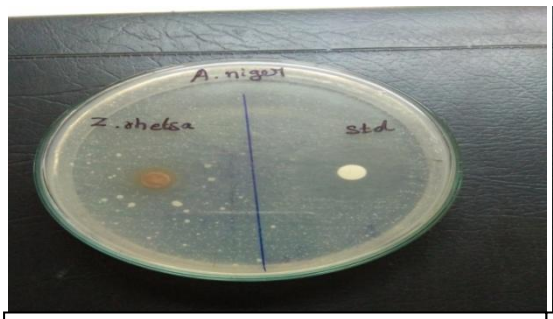
E.coli	*Pseudomonas auregenosa*

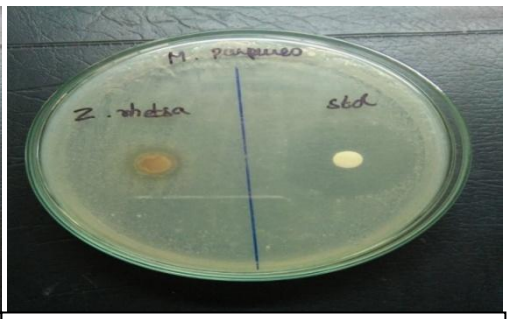
ANTIFUNGAL ACTIVITY OF AQZR

Table 16: Antifungal activity of AQZR

Sino	Organisms	Standard Fluconazole(mm)	AQZR(mm)
1	*Aspergillus niger*	21	12
2	*Monascus purpureus*	24	15

Figure 12: Antifungal activity of AQZR





Aspergillus niger	*Monascus purpureus*

6.6.1 MINIMUM INHIBITORY CONCENTRATION OF AQZR

BACTERIAL ORGANISMS

Table 17: MIC values of AQZR

SI NO	GRAM +VE & GRAM –VE ORGANISMS	MIC VALUE OF AQZR
1	*Bacillus subtilis* (µg/ml)	250
2	*Staphylococcus aureus* (µg/ml)	125
3	*E.coli* (µg/ml)	125
4	*Pseudomonas auregenosa* (µg/ml)	125

Figure 13: MIC of AQZR

FUNGAL ORGANISMS

Table 18: MIC values of AQZR

SI NO	FUNGAL ORGANISMS	MIC VALUE OF AQZR
1	*Aspergillus niger* (µg/ml)	250
2	*Monascus purpureus* (µg/ml)	250

Figure 14: MIC of AQZR

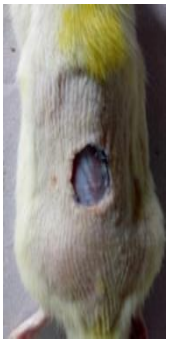
6.8 PHARMACOLOGICAL STUDIES

6.8.1 EVALUATION OF WOUND HEALING EFFECT OF AQZR IN EXCISION WOUND MODEL

Figure 15: Percentage wound contraction on excision wound model

On 1st day

Control



Simple base ointment

Standard

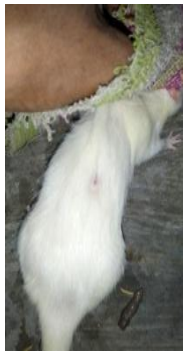
AQZR

On 21st day

Control

Simple base ointment

Standard



AQZR

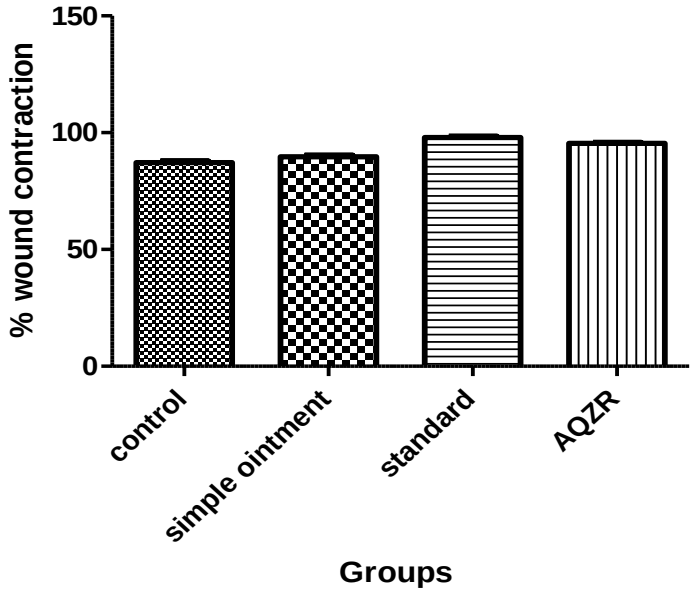
6.9. ESTIMATION OF PARAMETERS

6.9.1. PERCENTAGE WOUND CONTRACTION

Table 19: Percentage wound contraction in excision wound model

COMPOUNDS	0thDay	3rdDay	7thDay	13thDay	17thDay	19thDay
	% WC	% WC	% WC	% WC	% WC	% WC
CONTROL	0	27.5±0.58	23.6±2.03	74.2±0.99	72±0.99	85.1±0.99
SIMPLE BASE OINTMENT	0	13.5±0.49	27±0.83	52.5±1.24	66.8±1.24	88.2±1.88
STANDARD	0	15.7±0.29	39.8±0.77	81.8±1.09	88.5±1.25	99.5±1.10
AQZR OINTMENT	0	10±0.42	13.8±1.48	55.4±1.08	84.1±1.08	96.9±1.08

Figure 16: Effect of AQZR on % wound contraction in excision wound model



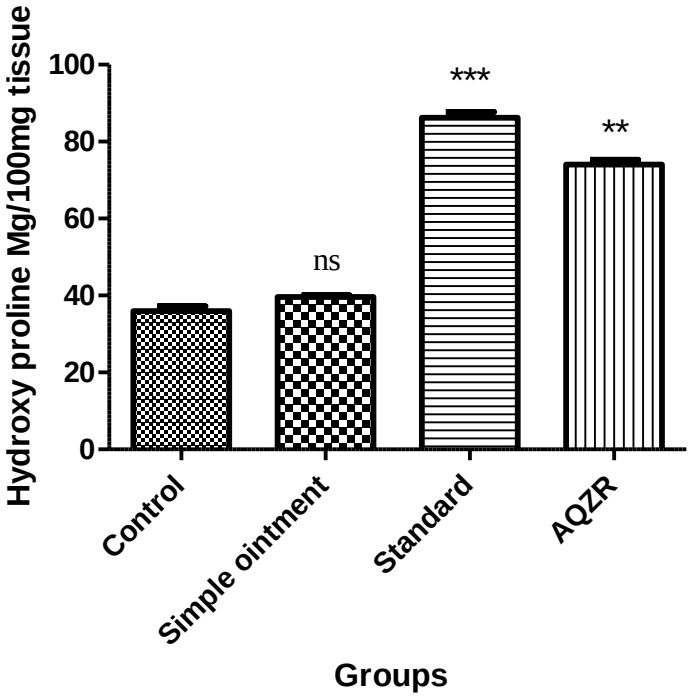
6.9.2 ESTIMATION OF HYDROXYPROLINE

Table 20: Hydroxyproline levels in excision wound models.

GROUP	HYDROXY PROLINE (µg/g)
Control	36.64±2.107
Simple base ointment	39.00±3.123^{ns}
Standard	81.12±3.142***
AQZR	70.11±2.010**

Statistical comparison Each group (n=5), each value represents mean ± SEM. One way Anova was performed $^{***}P<0.001$, $^{**}P<0.01$, $^{*}P<0.05$,when all groups compared with control.

Figure 17: Effect of AQZR on Hydroxyproline level in excision wound model



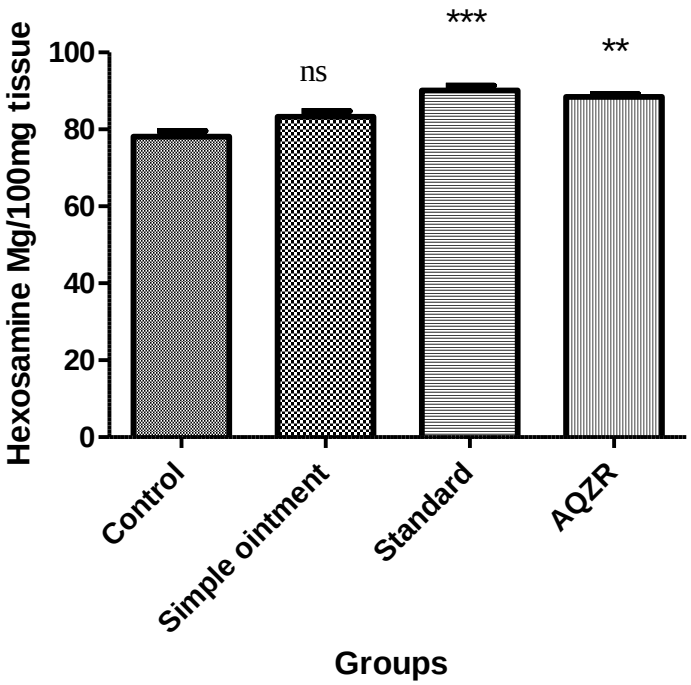
6.9.3 ESTIMATION OF HEXOSAMINE

Table 21: Hexosamine levels in excision wound model.

GROUP	HEXOSAMINE (µg/g)
Control	73.81±1.271
Simple base ointment	79.81±1.701 ^{ns}
Standard	88.12±1.412***
AQZR	85.10±1.812**

Statistical comparison Each group (n=5), each value represents mean ± SEM. One way Anova was performed ***P<0.001, **P<0.01, *P<0.05, when all groups compared with control.

Figure 18: Effect of AQZR on Hexosamine level in excision wound model



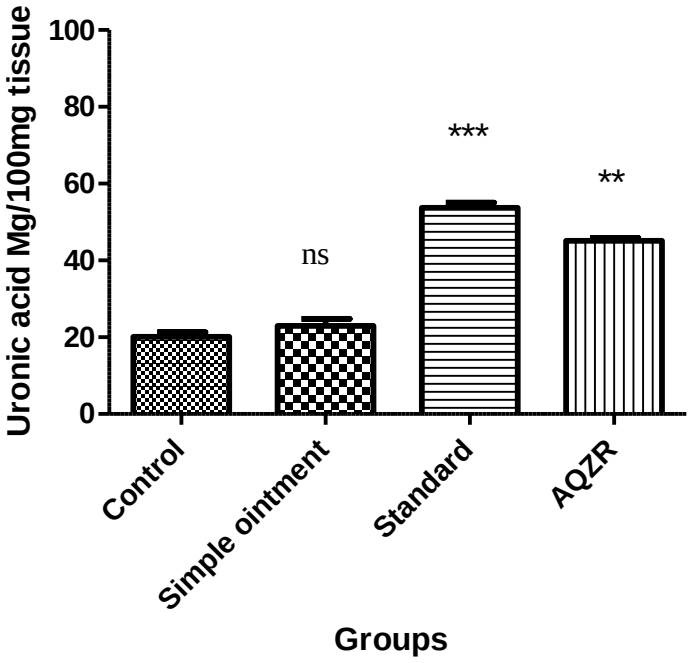
6.9.4. ESTIMATION OF URONIC ACID

Table 22: Uronic acid levels in excision wound model.

GROUP	URONIC ACID (µg/g)
Control	17.12±0.412
Simple base ointment	19.41±0.481 ^{ns}
Standard	50.17±0.644***
AQZR	42.49±0.556**

Statistical comparison Each group (n=5), each value represents mean ± SEM. One way Anova followed by Dunnets test was performed***P<0.001, **P<0.01, *P<0.05,when all groups compared with control.

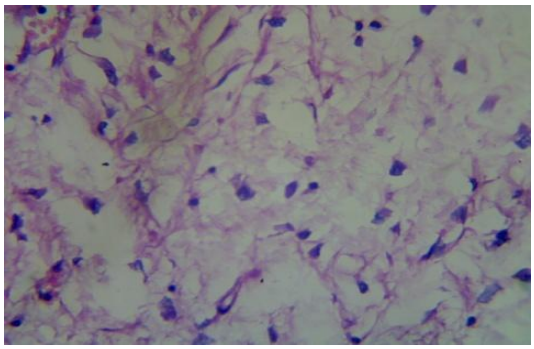
Figure 19: Effect of AQZR on Uronic acid level in excision wound model



6.10. HISTOPATHOLOGICAL STUDY

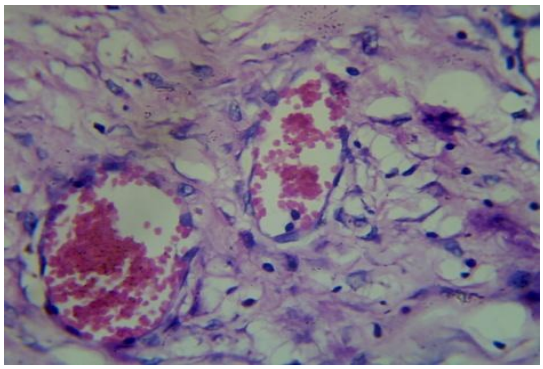
Histopathological evaluation of excision wound model.

Figure 20: Group 1- Control



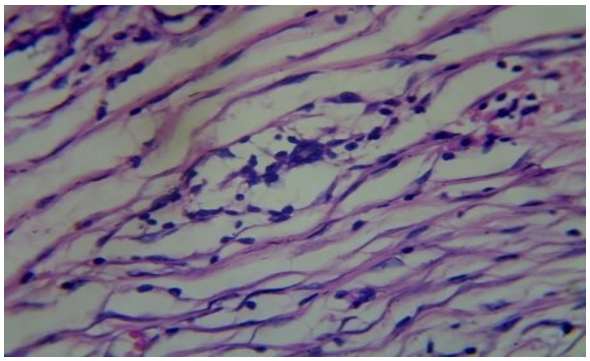
The section of the skin and epidermis shows incomplete healing with less epithelialization and lesser collagen formation indicated the incomplete wound healing.

Figure 21: Group 2- Simple base ointment



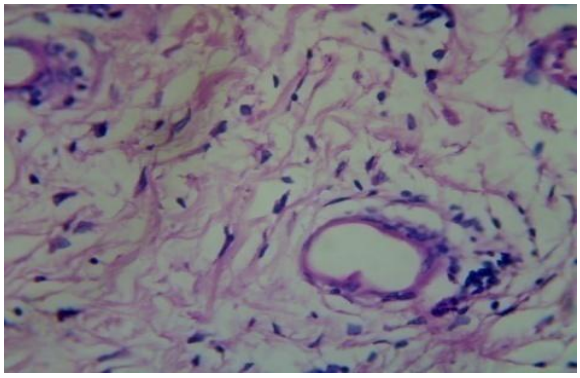
The section of the skin and epidermis shows incomplete healing. Section of the skin and epidermis shows proliferation of fibroblasts

Figure 22 : Group 3- Standard



Section of the epidermis shows Re-epithelialization with the proliferation of fibroblasts and few lymphocytes in the sub epithelium with thin walled congested blood vessels.

Figure 23: Group 4 - AQZR treated



Section of the epidermis shows Re-epithelialization with the proliferation of fibroblasts, of dense fibrous tissue and blood capillaries was observed. Few macrophages were also present.

7. DISCUSSION

Wound is a clinical entity and is as old as mankind, often considered as major problem in clinical practice. Each year, millions of people experience burns, suffer from chronic wounds, or have acute wounds that become complicated by infection, dehiscence or problematic scarring. Wounds are normally resolved in a few days, but chronic wounds represent a major burden because of economic and social factors. There by, the search for new agents is ongoing and natural products become a great target. Individual factors such as stress or diabetes can cause delays in the healing process or increase the risk of infection in the wound. Due to unawareness of the society this may lead to chronic diseases which may damage the other organs. Impaired wound healing can result into severe morbidity leading to long hospitalization of patients. States (US) population, which results in a significant economic burden estimated to be nearly 2–4% of the health budgets.

Zanthoxylum rhetsa (Roxb) DC the paradise tree has a long history in herbal medicine in various countries. The bark, seed, and stem extract of *Zanthoxylum rhetsa* is well known for its different types of pharmacological properties such as Digestive problem, Inflammatory dermatosis, Tooth ache, Cytotoxic properties, Antinociceptive, Antioxidant, Antibacterial, Antifungal. Externally it is used for wound healing. The major active constituents are budrungaine, budrungainine, rhetine, rhetsine, evodiamine, chelerythrine, lupeol. The present study was aimed to evaluate the wound healing potential of aqueous stem extract of *Zanthoxylum rhetsa* in excision wound model of rats.

Phytochemical screening of AQZR were performed and the results revealed the presence of alkaloids, proteins and amino acids, flavonoids, tannins, phenols, glycosides, tannins, carbohydrates. The main attraction of the phytochemical was the presence of phenols and flavonoids which was concluded by the colorimetric estimation of these constituents in the extract.

Flavonoids are coming under the category of polyphenols, where its action is mainly attributed to anti-inflammatory action and it provides a symptomatic relief in wound.

Phytopharmaceuticals are gaining importance in modern medicine as well as in traditional system of medicine owing to their therapeutic effect due to the presence of phytochemicals such as polyphenols, flavonoids, terpenoids etc.

Flavonoids are wide spread plant secondary metabolites that have shown free radical scavenging activity and protection against oxidative stress. Studies were revealed that flavonoids are also known to promote the wound healing process mainly due to their astringent and antimicrobial properties which appear to be responsible for the wound healing and increased rate of epithelialization. Polyphenols are the major plant compounds with antioxidant activity that is mainly due to their redox properties which enables them to act as reducing agents, hydrogen donors and singlet and triplet oxygen scavengers. Results obtained in the present study revealed that the levels of these phytoconstituents were considerable and the total phenol and flavonoid content was found to AQZR-71.37mg/g and AQZR-40.64mg/g respectively.

Antioxidants are compounds that inhibit or delay the oxidation of other molecules by inhibiting the initiation or propagation of oxidizing chain reactions. They are radical scavengers which protect the human body against free radicals that may cause pathological conditions such as ageing, dementia, cancer etc. Therefore in the present study, the potential of the AQZR to serve as antioxidants was assayed.

Free-radicals play an important role in the oxidative damage of biological systems. Several complementary methods have been adopted to trap free radicals through antioxidant activity, among which DPPH* assay is the most common. The effect of antioxidants on DPPH is thought to be due to their hydrogen donating ability. The free radical scavenging activity of the extract was estimated by comparing the % inhibition of AQZR with standard Quercetin. IC_{50} was also calculated to determine the amount of extract needed to quench 50% of radicals. Stem extract of *Zanthoxylum rhetsa* exhibited

a dose dependent scavenging activity with IC_{50} values of, 17.77 µg/ml respectively for AQZR. Where the IC_{50} for standard Quercetin was found to be 1.55 µg/ml.

The ABTS+ scavenging assay, which employs a specific absorbance (734nm) at a wavelength remote from the visible region and requires a short reaction time, can be used as an index that reflects the antioxidant activity of the test samples. The presence of specific chemical compounds in the extracts of *Zanthoxylum rhetsa* may inhibit the potassium persulfate activity and hence reduced the production of ABTS+. *Zanthoxylum rhetsa* extracts were found to be effective in scavenging radicals and the increase was concentration-dependent. IC_{50} value of Quercetin was 0.1142 µg/ml whereas 1.160 µg/ml for AQZR respectively. This shows that *Zanthoxylum rhetsa* stem extract presents a good ability to scavenge the ABTS radical.

The NO scavenging assay, which employs a specific absorbance (546nm) at a wavelength remote from the visible region and requires a short reaction time, can be used as an index that reflects the antioxidant activity of the test samples. *Zanthoxylum rhetsa* extracts were found to be effective in scavenging radicals and the increase was concentration-dependent. IC_{50} value of Quercetin was 1.55µg/ml whereas 30.5 µg/ml for AQZR respectively. This shows that *Zanthoxylum rhetsa* extract presents a good ability to scavenge the NO radical. The antioxidant activities against ABTS, DPPH and NO were correlated with the concentration, chemical structures, and polymerization degrees of organ antioxidants.

Thus from the result obtained it could be concluded that AQZR shows a good antioxidant activity which might be attributed to the presence of phytochemicals such as polyphenols and flavonoids.

The antimicrobial study was carried out aqueous extract of *Zanthoxylum rhetsa* against different strain of bacteria (2 Gram + ve and 2 Gram – ve) and fungi (2 strains), that are known to cause infection in human and plants, by disc diffusion method at 200µg/disc. The ciprofloxacin 10µg/disc and fluconazole 10µg/disc were used as standard for bacteria and fungi respectively.

The standard ciprofloxacin and fluconazole was found to have significant antimicrobial activity against bacteria and fungi respectively. The various zones of inhibition were observed from all the extracts against various strains. Among the extracts the AQZR was observed to have significant antimicrobial activity. The zone of inhibition was observed from Gram +ve and Gram –ve bacteria and fungi strains. The maximum zone of inhibition (14mm) was found in AQZR of *Zanthoxylum rhetsa* against *Staphylococcus aureus* for the Gram +ve organism and for Gram –ve organisms the maximum zone of inhibition (10mm) was found in AQZR against *Pseudomonas auregenosa.*

The extract showed antifungal activity, the antifungal activity was observed maximum in AQZR. Among all, the zone of inhibition was found to be maximum for *Monascus purpureus* (15mm).

Minimum Inhibitory Concentration (MIC) is defined as the highest dilution or least concentration of the extracts that inhibit growth of organisms. Determination of the MIC is important in diagnostic laboratories because it helps in confirming resistance of microorganism to an antimicrobial agent and it also monitors the activity of new antimicrobial agents.

For bacterial and fungal MIC study, AQZR showed least MIC value i.e. 125µg/ml against *Staphylococcus aureus, E.coli, Pseudomonas auregenosa*. The AQZR showed MIC value i.e. 250 µg/ml against *Bacillus subtilis, Aspergillus niger, Monascus purpureus.*

Aqueous stem extract of *Zanthoxylum rhetsa* demonstrated wound healing properties comparable with that of antibiotic standard. Animals in the untreated group showed some degree of healing. As earlier suggested, healing in this untreated group may be due to self-immunity. It is important to note that throughout the period of wound treatment, the extracts did not cause irritation or pain to the animals as the rats neither show any signs of restlessness nor scratching/biting of wound site when the extract were applied.

In this investigation three models were used to assess the effect of the AQZR extracts as applied topically. The plant may have a beneficial influence on the various phases of wound healing like fibroplasias, collagen synthesis, and wound contraction, resulting in faster healing. The results of the present investigation showed that both plant extracts have definite wound healing action.

Extract treated excision wounds showed an increased rate of wound contraction, leading to faster healing as confirmed by the increased healed area when compared to the control untreated group. The AQZR was recorded similar effectiveness when compared to the group treated with a commercial brand of 5% povidone iodine ointment in but the magnitude was lesser than standard.

Increased wound contraction rate was also observed in the extract treated excision wounds, when compared to control. Povidone iodine ointment was used as a standard, the rate of healing was slightly more than the test AQZR.

Measurement of the hydroxyproline could be used as an index for collagen turnover. Collagen is a major protein of the extracellular matrix and is the major component that ultimately contributes to wound strength. Breakdown of collagen liberates free hydroxyproline and its peptides. It was observed that *Zanthoxylum rhetsa* increased the collagen content of the skin ultimately and contributed to wound strength. Previous studies stated that there is a strong correlation between the collagen fiber formation and acceleration of wound healing.

From histopathological studies, it was observed that the phases of wound healing occurred in a timely manner. The untreated (control) wound healed slowly when compared to the wounds treated with AQZR. Extract treated group shows large number of collagen tissue (fibrosis) and neovascularisation with minimal inflammatory cells. AQZR treated showed near to normal features, collagen tissue (fibrosis), and neovascularisation when compared to control group.

8. CONCLUSION

Wound healing is a complex and continuous process that begins immediately after injury, followed by homeostasis, blood clotting, inflammation, proliferation and remodeling phases. All these phases can promote or prolong healing by influencing external or internal factors including infection sex hormones and nutrition. Delay in healing process increases the possibility of getting infected, improper recovery, and formation of unpleasant scar.

The study thus demonstrated the wound healing activity of aqueous stem extract of *Zanthoxylum rhetsa* (Roxb) DC and found to be effective in the functional recovery of the wound. The extracts promote wound contraction; increases hydroxyproline, hexosamine and uronic acid of excision wound as compared to control group. The result may be attributed to the phytoconstituents such as flavonoids and phenolics present in it which may be due to their individual or cumulative effect that enhanced wound healing and provided scientific evidence to the ethnomedicinal futures of *Zanthoxylum rhetsa*(Roxb) DC. These findings could justify the inclusion of this plant in the management of wound healing.

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